Botulinum toxin modulation of mouse bladder sensory afferent signalling

by

Hodan Ibrahim

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Abstract

This thesis is an investigation of the effects of botulinum neurotoxins (BoNTs) on the ability of the sensory nerves innervating the bladder to detect and transduce mechanical and chemical stimuli. The healthy functioning of the bladder is dependent on the sensory limb of the micturition cycle, and hyperactivation may lead to the development of urgency, a symptom associated with overactive bladder (OAB). Currently, the treatment options for patients with OAB are not ideal, however, injections of BoNT serotype A is a well-tolerated and effective third line treatment. The mechanisms behind the BoNT/A dependent reduction in urgency symptoms are not well understood, and it is unclear how a neurotoxin best known for paralysing the neuromuscular junction may affect the ability of sensory nerves to detect stretch during filling.

Using a well-characterised *ex vivo* mouse electrophysiology preparation where the responses of afferent nerves to bladder filling are recorded directly, a wide variety of BoNT serotypes and recombinant constructs were investigated. In **chapter three**, studies into the entry mechanisms of BoNT/A into the bladder wall revealed the well-establised double-receptor mechanism was not necessary for internalisation, as the light chain (LC/A) alone significantly inhibited afferent neurotransmission. In **chapter four**, two other serotypes of BoNT, BoNT/B and BoNT/E were investigated, to understand whether modulation of afferent neurotransmission was a feature specific to BoNT/A. These experiments revealed both BoNTs /B and /E directly inhibited distension-induced firing. In **chapter five**, the role of SNARE cleavage on BoNT/A induced afferent inhibition was investigated using the catalytically inactive construct BoNT/A (0) unable to cleave SNAP-25. This construct had no effect on hemidiaphragm contractility yet potently inhibited distension induced afferent firing, and was more effective at doing so than catalytically active BoNT/A. These findings are novel and reveal a SNAP-25 independent modulation of sensory signalling.

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Article

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Oral presentation

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List of abbreviations

ACh	Acetylcholine
AM	Antimuscarinic
ASIC	Acid sensing ion channel
ATP	Adenosine triphosphate
AUM	Asymmetric unit membrane
BOO	bladder outlet obstruction
CB	Cannabinoid
CGRP	Calcitonin related gene peptide
DAG	Diacylglycerol
DRG	Dorsal root ganglia
ENaC	Epithelial Na+ channel
EP	Prostaglandin receptor
FGFR	Fibroblast growth receptor 3
GAG	Glycosaminoglycan
GD	Disialogangliosides
cGMP	Cyclic guanosine monophosphate
GT	Trisialogangliosides
GTP	Guanosine-5'-triphosphate
HA	Haemagglutinin
HC	Heavy chain
HN	N-terminus of the heavy chain
5-HT	5-hydroxytryptamine
IC	Interstitial cell
ICC	Interstitial cells of Cajal
IC/PBS	Interstitial cystitis/Painful bladder syndrome
IDO	Idiopathic detrusor overactivity
IF	Immunofluorescence
IHC	Immunohistochemistry
IP3	Inositol trisphosphate
IR	Immunoreactivity
LC	Light chain
LD$_{50}$	Lethal dose, 50%
LPS	Lipopolysaccharide
LUT	Lower urinary tract
MLCK	Myosin light chain kinase
MLCP	Myosin light chain phosphatase
NANC	Non-adrenergic non-cholinergic

NDO	Neurogenic detrusor overactivity
NGF	Nerve growth factor
NHS	National health service
NICE	National institute for health and care excellence
NK	Neurokinin
NMJ	Neuromuscular junction
BoNT	Botulinum neurotoxin
NTNH	Non-toxic non-haemagglutinin
OAB	Overactive bladder
PACAP	Pituitary adenylate-cyclase-activating polypeptide
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PSG	Polysialogangliosides
PTC	Progenitor toxin complex
SNAP-25	Synaptosomal-associated protein 25
cSNAP-25	Cleaved SNAP-25
SNARE	Soluble N-ethylmaleimide sensitive factor attachment protein receptor
SOC	Store operated Ca^{2+}
SV2	Synaptic vesicle protein 2
TH	Tyrosine hydroxylase
TRP	Transient receptor potential
TRPA	Transient receptor potential ankyrin
TRPM	Transient receptor potential melastatin
TRPV	Transient receptor potential vanilloid
UTI	Urinary tract infection
VAChT	Vesicular acetylcholine transporter
VAMP	Vesicle associated membrane protein
VGCC	Voltage gated calcium channel
VIP	Vasoactive intestinal peptide

CHAPTER 1 - GENERAL INTRODUCTION

1.1 Lower urinary tract anatomy

The lower urinary tract is responsible for the collection and storage of urine through the coordinated action of the urinary bladder and urethra, which expel it from the body at a socially convenient time. The bladder is a hollow, distensible, collapsible, muscular organ, which sits in the pelvic cavity posterior to the pubic symphysis. The kidneys produce urine by filtering excess water and waste from the bloodstream, which continuously flows down the ureters and is collected in the bladder (see figure 1). A healthy adult bladder typically collects 300-500 ml of liquid, as its distensibility allows storage of large volumes at low pressure (Lukacz et al., 2011).

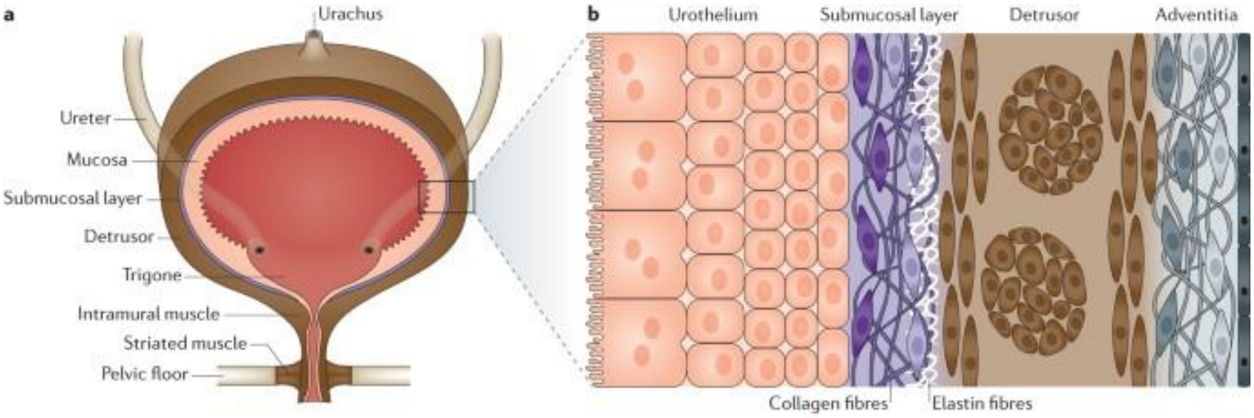


Figure 1.1: A diagram of the anatomy of the bladder, describing the anatomical regions as well as a cross-section of the bladder wall. Adapted from Ajalloueian et al (2018).

The bladder is organised anatomically and functionally into three regions

1) Dome – the apex of the bladder pointing up towards the abdomen, composed of smooth muscle
2) Trigone – the triangular shaped region at the base of the bladder, outlined by the ureter entry points and the bladder neck

3) Neck – connection between the bladder and the urethra

Histologically, the bladder wall is organised into four main layers, the lumen is lined with an epithelial layer, termed the urothelium, followed by the submucosal layer, the detrusor layer and the adventitia. These are described in further detail below.

1.2 Structure and function of the bladder wall

The urothelium

- Structure

The urothelium lines the luminal side of the bladder wall and is composed of a stratified transitional epithelium with three distinct cell layers, the apical umbrella, intermediate and basal cell layers. The uppermost umbrella cells are highly specialised to provide an impermeable barrier between the urine in the lumen and the bloodstream. The multiple features that facilitate the maintenance of the barrier include expression of tight junctions, uroplakins, and a glycosaminoglycan (GAG) layer. The tight junction proteins are expressed between the umbrella cells and the intermediate cells, providing defense against the waste products in the urine, which could lead to the development of inflammation and cystitis if allowed to breach the barrier (Kreft et al., 2005; Lavelle et al., 2000). The GAG layer has also been shown to play a role in maintaining the barrier function of the urothelium, Hurst *et al* (2016) demonstrated that digestion of the GAG layer in rats led to increased permeability without damage to the urothelium (Hurst et al., 2016).

The intermediate cell layer of the urothelium lies between the apical umbrella cells and the basal cells, they are partially differentiated and develop to become umbrella cells when the previous umbrella cells are lost. As the urothelium exhibits low turnover, terminal differentiation occurs only when there is an infection or injury to kickstart regeneration (Koprivec et al., 2001). The basal cells form a single cell layer connecting the urothelium to the lamina propria, where almost all cell division occurs (Kurzrock et al., 2008; Library et al., 2011; Papafotiou et al., 2016). A population of basal cells that give rise to all cell types of the urothelium during regeneration are considered to be urothelial stem cells and have been implicated in bladder cancer as they express markers found in urothelial tumours (Papafotiou et al., 2016).

- Barrier function

Maintenance of an impermeable barrier is a critical component to healthy bladder function, as the urothelial cells make direct contacts with urine and waste products within it. Barrier function is maintained through several features specific to the umbrella cells, including tight junctions, lipids and uroplakins. Uroplakins are highly conserved transmembrane proteins that form a physical barrier on top of the umbrella cells, to create an asymmetric unit membrane (AUM) (Yu et al., 1994). The importance of uroplakins to the healthy functioning of the bladder has been illustrated using knock out mice, Hu *et al* (2000) found mice without the uroplakin III gene exhibited increased expression of uroplakin Ib, which usually complexes with uroplakin III in the AUM (Deng et al., 2002; Hu et al., 2000). This suggests overcompensation for the loss of uroplakin III. Urothelial cells without uroplakin III exhibited smaller plaques and increased permeability, as well as gross abnormalities in upper urinary tract function such as hydronephrosis, altered renal function and enlarged ureters (Hu et al., 2000).

When the bladder is empty, the umbrella cells take a cuboidal shape, while in filled bladders they become stretched and appear squamous (Truschel et al., 2002). To accommodate increasing volumes, umbrella cells increase surface area by releasing uroplakin-containing fusiform vesicles. As the bladder contracts to empty, the process of compensatory endocytosis is initiated to recover that extra membrane (Khandelwal et al., 2010). The mechanism underlying the exocytosis of the fusiform vesicles is a systematic process controlled by Rab GTPases which regulate vesicular release through SNARE proteins (Born et al., 2003; Khandelwal et al., 2008; Wankel et al., 2016). Wang *et al* (2003) described the effect of mechanical stretch on the surface area of umbrella cells, using modified Ussing chambers they applied hydrostatic pressure on the urothelium and found that transepithelial capacitance, a measure of surface area, increased as pressure increased

(Wang et al., 2003). This was further expanded on by Yu *et al* (2009) who showed that it was not necessarily pressure itself, but the increased stretch applied to the membrane and concomitant Ca^{2+} influx that initiated the release of fusiform vesicles (Yu et al., 2009). These findings suggest that the urothelium is well adapted to not only providing an impermeable barrier to waste products in the urine, it may also respond to increased stretch imposed by bladder filling.

- Sensory signalling

The input of sensory information is crucial for the coordination of the micturition cycle. In recent decades, evidence suggesting a role for the urothelium in the transduction of mechanical stretch during bladder filling showed that the generation of sensory input was more complicated than previously thought. In response to mechanical stimulation, the urothelium releases a variety of neurotransmitters such as adenosine triphosphate (ATP), acetylcholine (ACh), adenosine, nitric oxide and prostaglandins (Ferguson, et al., 1997; Birder et al., 1998; Gillespie et al., 2005; Yu et al., 2006; Stromberga et al., 2020). The urothelium also expresses a wide range of receptors including stretch sensitive receptors, muscarinic, purinergic, adrenergic, TRP and bradykinin receptors (Carneiro et al., 2014; Chess-Williams, 2002; Chess-Williams et al., 2019; Grundy, Chess-Williams, et al., 2018; Ishihama et al., 2006; Moro et al., 2011; Ochodnický et al., 2012, 2013; Tempest et al., 2004; Vlaskovska et al., 2001). Studies have shown the urothelium is capable of responding to chemical stimuli, such as the bacterial lipopolysaccharide (LPS), to signal the presence of bacteria which may lead to the development of urinary tract infection (Silberfeld et al., 2020; Ueda et al., 2020). These findings have all led to the understanding that the urothelium is more than an inert layer of cells and instead can both detect sensory signals and respond through the release of neurotransmitters (see figure 1.2). Apodaca *et al* (2007) proposed that the urothelium, along with the suburothelial primary afferent and efferent nerve terminals and the interstitial cells, form a ‘sensory web’ where cells communicate through the release and detection of neurotransmitters and chemical mediators (Apodaca et al., 2007).

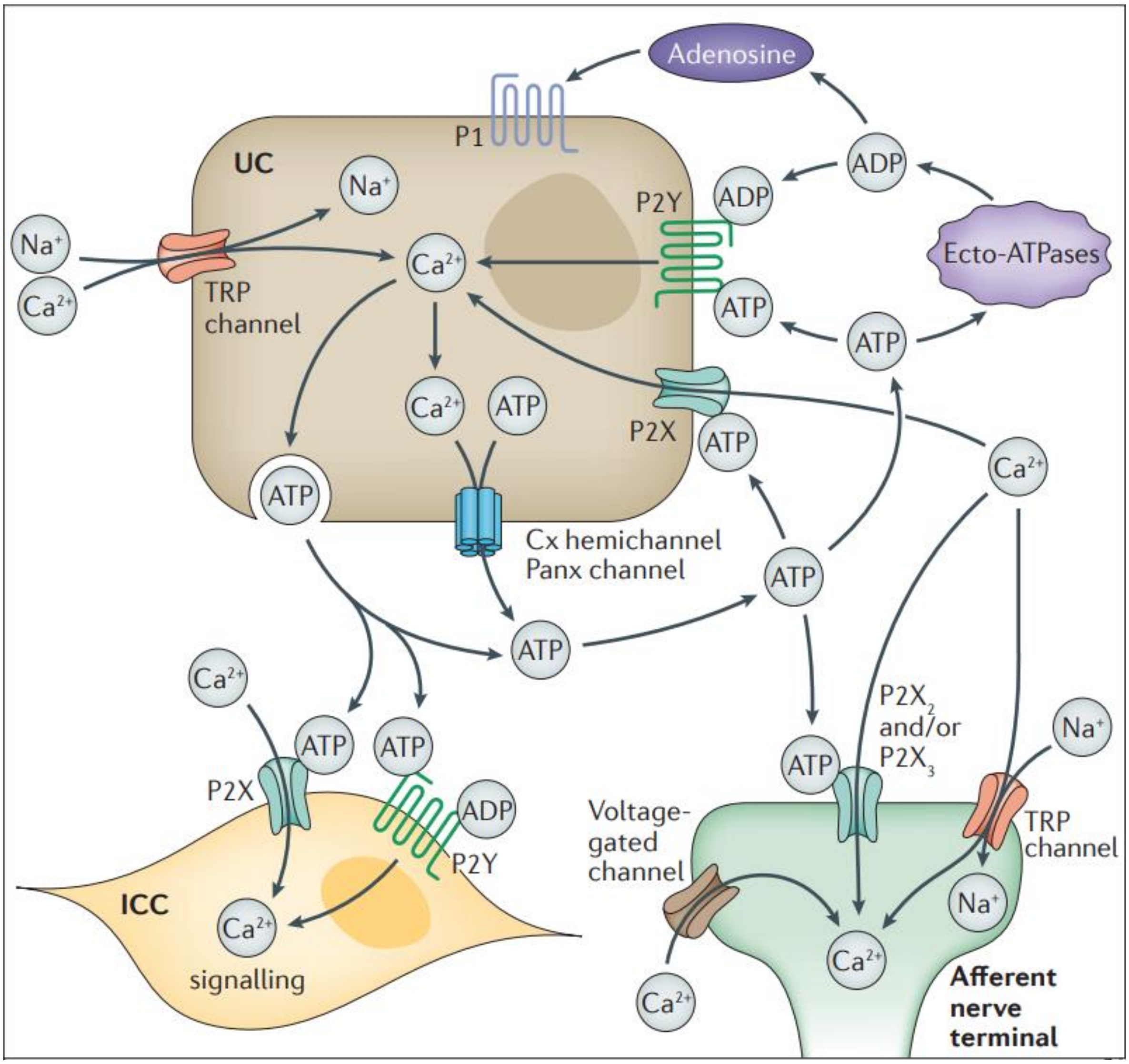


Figure 1.2: A diagram showing the proposed urothelial associated sensory web and the interactions associated with purinergic mechanosensation. Distension may activate TRPV4 channels which cause an influx of Ca^{2+} and Na^{+} into the urothelium, which consequently leads to ATP released through vesicles or connexin/pannexin hemichannels. Purinergic receptors on the interstitial cells or afferent terminals propogate that signal further. ATP may also be degraded by ectonucleutidases (Ecto-ATPases) to produce adenosine, which acts on P1 receptors to potentially modulate ATP release. Adapted from Merrill et al. (2016).

The lamina propria

The submucosal layer, or the lamina propria, is located in between the basement membrane of the urothelium and the detrusor smooth muscle and is split into two parts, the cellular component immediately below the urothelium, which contains interstitial cells, afferent and efferent nerves, fibroblasts, and blood vessels. The second, more fibrous component is rich in collagen fibres and smooth muscle fascicles to provide structural support (Gabella, 2019). Recent work has shown that the lamina propria has many roles in maintaining healthy bladder function, including coordination of sensory signalling, maintaining bladder elasticity, compliance and contractility (Andersson & McCloskey, 2014).

Interstitial cells (ICs) are present in the lamina propria and the detrusor, in the literature they have also been named interstitial cells of Cajal (ICCs), telocytes, fibroblast-like cells and myofibroblasts. The presence of interstitial cells in the bladder wall was first described by Smet *et al* (1996), after they found immunoreactivity of cGMP in cells they described to be of mesenchymal origin and appeared similar to the well characterised ICCs in the gut (Smet et al., 1996). Gut ICCs play an essential role as electrical pacemakers to maintain rhythmic smooth muscle contraction (Klein et al., 2013), which opened the possibility that the presence of these cells in the bladder wall could play a part in the initiation of bladder contractility. Shafik *et al* (2004) conducted immunohistochemistry of IC markers on human bladder tissue, concentrating on the bladder dome. They found aggregation of IC cells in the dome and suggested these cells could act as pacemakers, stimulating contraction during micturition (Shafik et al., 2004).

There is evidence that the lamina propria can contract following treatment with various stimuli, as investigators have found that urothelium/lamina propria only bladder strips exhibited contractility independent of the detrusor (Moro et al., 2011; Moro & Chess-Williams, 2012; Sadananda et al., 2008). The mechanisms behind these responses are still unclear, however in their review on the subject Chai *et al* (2016) suggest that due to the presence of smooth muscle in the porcine lamina propria, this could be the source of the recorded contractions (Chai et al., 2016).

The extensive vasculature of the lamina propria has received little attention, despite its importance. The dense plexus of capillaries in the lamina propria has connections with the basal cell layer of the urothelium and is organised to avoid loss of blood flow during bladder distension (Miodon´ski et al., 1999). Shimizu *et al* (2014) showed that suburothelial blood vessels responded to sympathetic neural activity from the corresponding nerve fibres with contraction, which were blocked by α-adrenoreceptor antagonists and β3 agonists to induce relaxation (Shimizu et al., 2014). Interestingly, the compounds tested are also used to treat overactive bladder, which means that perhaps they could also be acting on the suburothelial microvasculature.

The detrusor

The bladder smooth muscle is known as the detrusor, and morphologically can be split into three layers; two longitudinal layers with an inner circular layer between them. The thickness of the smooth muscle is higher in the dome of the bladder compared to the trigone, surrounded by connective tissue fibres and networks of interstitial cells.

The smooth muscle cells of the detrusor are spindle shaped with a size of around 200µm and contain the contractile filaments actin and myosin. The cells are connected mechanically and electrically, as gap junctions and dense bodies distribute ions and force respectively. The thin actin filaments are attached to the dense bodies, where thick myosin filaments are able to bind during contraction (Fry et al., 2010)

Contraction is initiated by release of ACh from parasympathetic nerve terminals, which activates muscarinic M3 G-protein coupled receptors (see figure 1.3). This leads to the activation of phospholipase 3 which targets phosphatidylinositol-4,5-bisphosphate (PIP2), which is then hydrolysed into diacylglycerol (DAG) and inositol triphosphate (IP3). These molecules act on the

L-type Ca^{2} channels and sarcoplasmic reticulum to release extracellular and intracellular Ca^{2+} respectively. The Ca^{2} ions bind to calmodulin to create a complex and activate the myosin light chain kinase (MLCK) to phosphorylate myosin and induce contraction (Fry et al., 2010). Relaxation is induced following dephosphorylation of myosin by the myosin light chain phosphatase (MLCP), which means smooth muscle contractility is under tight control and depends on MLCK and MLCP concentrations.

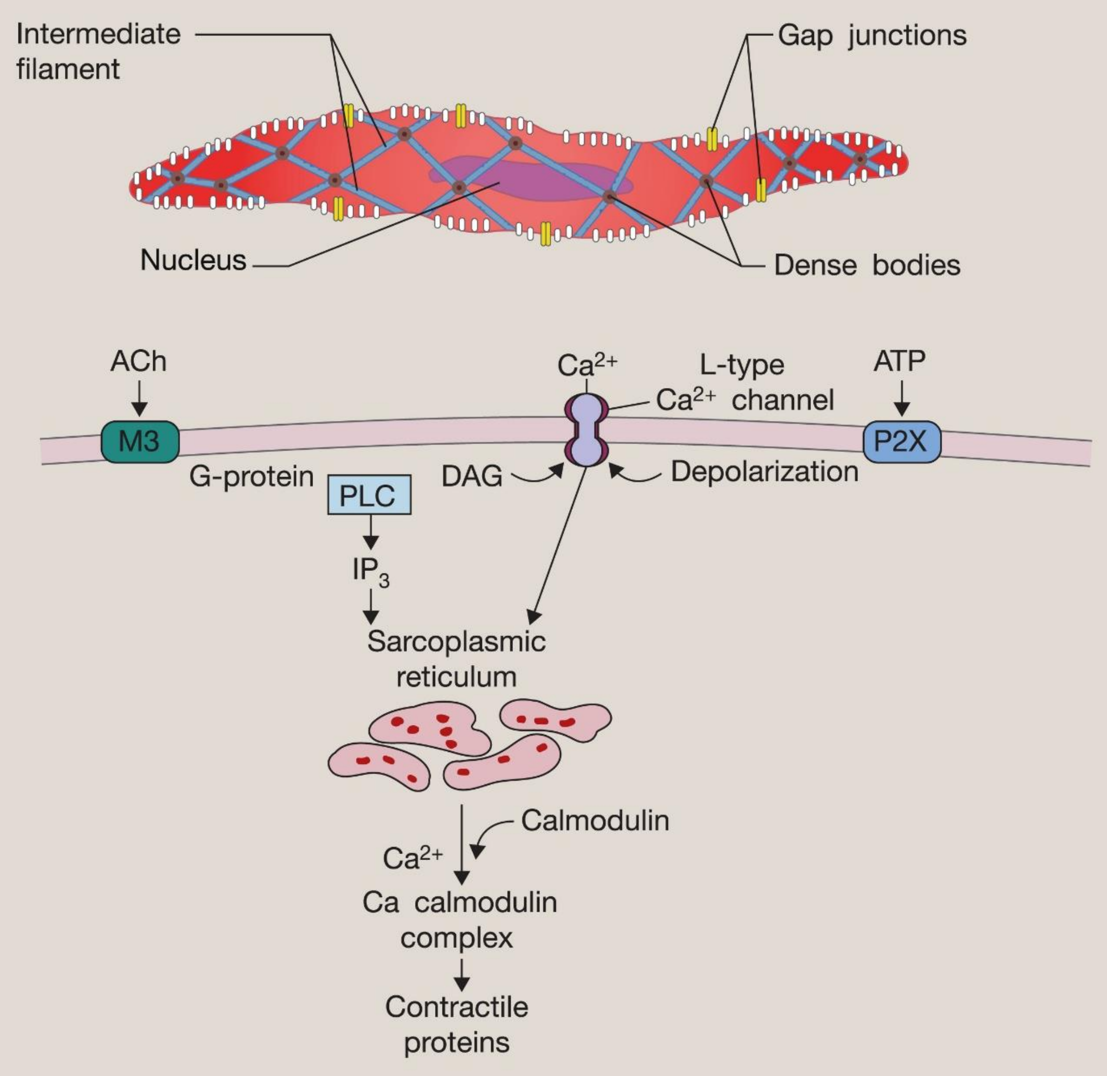


Figure 1.3: A diagram showing an example of a smooth muscle cell (above) and the mechanism of contraction (below). Adapted from Sekhon & Ballaro (2022).

1.3 Bladder neurophysiology

Continence is achieved through the complex integration of the involuntary autonomic motor system and voluntary somatic motor system that allows us to choose to micturate at socially acceptable times. Their function is informed by the sensory afferent nerves, which constantly monitor the level of bladder fullness. The bladder is innervated by three groups of peripheral nerves; the sacral parasympathetic nerves (pelvic nerves), thoracolumbar sympathetic nerves (hypogastric nerves) and sacral somatic nerves (pudendal nerves) (Yoshimura & de Groat, 1997). These nerves are mixed, containing both afferent and efferent fibres. Micturition reflexes are coordinated by the pontine micturition reflex, which has connections to the cerebral cortex to facilitate conscious decision-making about voiding (see figure 1.4).

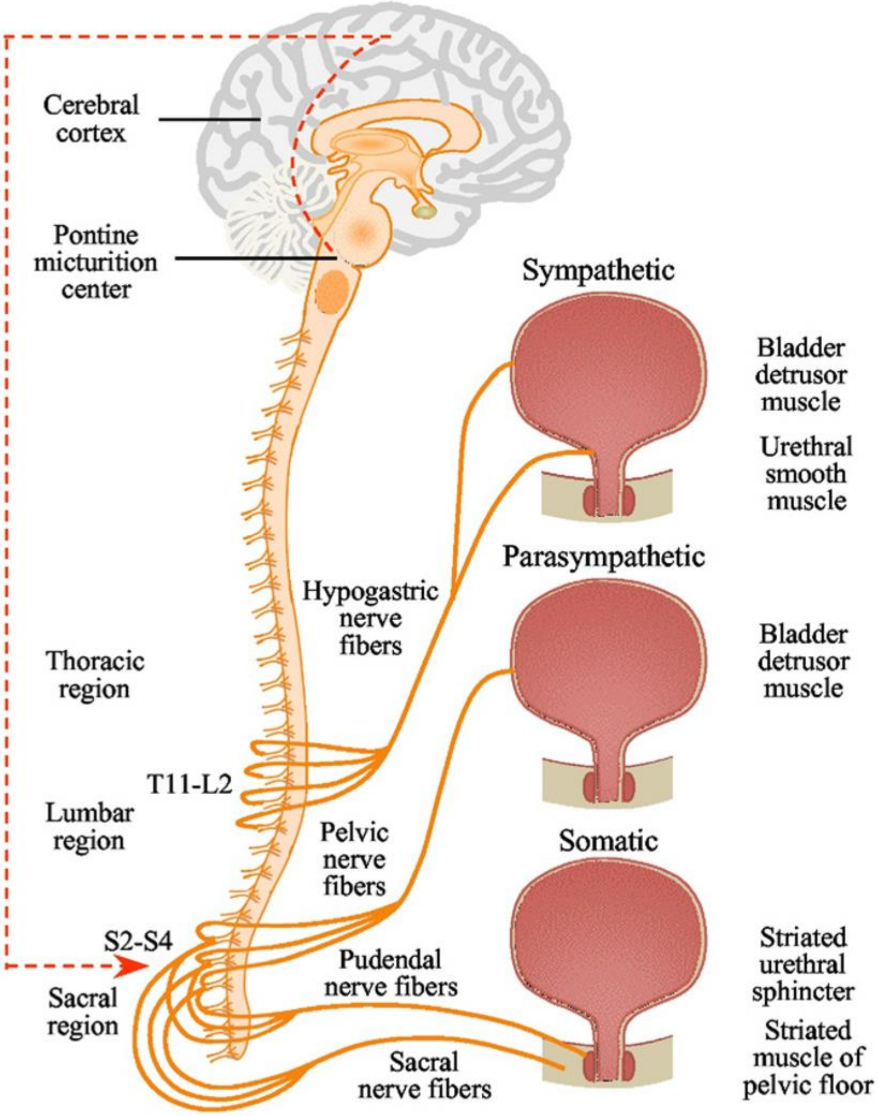


Figure 1.4: a visualization of the human lower urinary tract and its innervation. The bladder and its outlet, which includes the bladder neck and the internal and external urethral sphincters, are coordinated by the hypogastric (sympathetic), pelvic (parasympathetic) and pudendal (somatic) nerves. Hypogastric fibres originate in the T11-L2 segment of the spinal cord, while the pelvic and pudendal fibres originate in the sacral S2-S4 region. Adapted from Kanai & Andersson (2010).

Efferent innervation

Excitatory input to the bladder is controlled primarily by the parasympathetic efferent pathway. In the spinal cord, preganglionic axons from the S2 to S4 column terminate in the pelvic plexus and the bladder wall. Acetylcholine is released by preganglionic neurons to act on nicotinic receptors on postganglionic neurons, which subsequently releases excitatory neurotransmitters to act on the detrusor smooth muscle, resulting in contraction (Yoshimura & de Groat, 1997).

The postganglionic efferent nerves are differentiated into sympathetic and parasympathetic fibres that release different neurotransmitters to modulate detrusor smooth muscle function. Early research found that bladder contraction is induced by cholinergic and non-adrenergic, non-cholinergic (NANC) mechanisms, and relaxation mediated by adrenergic mechanisms (Ambache & Zar, 1970). Cholinergic stimulation has been shown to be the most important pathway for inducing muscle contraction, as the M_3 receptor is responsible for the initiation of voiding (Igawa et al., 2004). The M_2 receptor appears to have little to no contribution in the initiation of contraction, which is interesting as it is more highly expressed (70%) than the M_3 receptor (30%) in the bladder (Matsui et al., 2002; Wang et al., 1995). Early research found that nerve mediated contractility remained following treatment with the muscarinic antagonist atropine (Ambache & Aboo Zar, 1970; Langley & Anderson, 1895; Ursillo, 1961), which was later discovered to be of purinergic origin by Burnstock *et al* in the 1970s (Burnstock, 1972; Burnstock et al., 1972; Kasakov & Burnstock, 1982). ATP is a cotransmitter released by the parasympathetic postganglionic neurons and acts on P2X receptors on the detrusor to induce contraction (Burnstock & Kennedy, 2011).

The sympathetic preganglionic nerves originate in the T11 to L2 column of the spinal cord and connect with postganglionic axons in the inferior mesenteric ganglion, the paravertebral ganglia and the pelvic ganglia (Yoshimura & de Groat, 1997). The hypogastric nerves release noradrenaline, which induces contraction in the bladder neck and urethra and relaxation in the bladder dome through the activation of the α_1-adrenoreceptors and the β_3-adrenoreceptors respectively (Yamaguchi & Chapple, 2007). The somatic pudendal nerves innervate the urethra and control the function of the external striated urethral sphincter, as well as the pelvic floor.

Afferent innervation

As the bladder fills, the afferent nerves transduce the physical stretch of the bladder wall into electrical impulses which are sent through the dorsal spinal cord to the brain. This system is essential for the maintenance of social continence, as the afferent signals inform conscious decision making into voiding. The cell bodies of afferent fibres are within the dorsal root ganglia (DRG), at the thoracolumbar and lumbosacral levels of the spinal cord (de Groat et al., 2015). The majority of afferent input to the spinal cord occurs through the lumbosacral spinal cord, as shown by previous studies that have used neuronal activation markers such as c-fos expression following bladder distension finding significant activation in this region (Vizzard, 2000).

A detailed analysis of the distribution of afferent nerves in the bladder wall was performed by Gabella and Davis (1998), by staining for the neuropeptide calcitonin gene-related peptide (CGRP). They showed that afferent innervation in the bladder wall was most predominant in the suburothelial plexus, with axons reaching up into the urothelial layer, along the smooth muscle and around blood vessels (Gabella & Davis, 1998). The majority of afferent innervation lied in the bladder base and trigone, which become sparser closer to the equator and the dome of the bladder (Gabella & Davis, 1998). These findings suggest sensory afferent neurotransmission is most predominant in the bladder base.

The bladder is innervated by lumbar splanchnic and pelvic nerves, which are subcategorized into myelinated (Aδ) and unmyelinated (C) fibres. Although previously it was thought that Aδ and C fibres differentially detect low and high threshold stimulation, multiple authors have found no relationship between mechanosensory threshold and conduction velocity (Sengupta & Gebhart, 1994; Shea et al., 2000; Su et al., 1997). Interestingly, Shea *et* al (2000) found that half of the pelvic C fiber mechanoreceptors they recorded exhibited low activation thresholds less than 10 mmHg (Shea et al., 2000).

Primary afferent nerves terminate at multiple levels within the bladder wall, which has been shown to affect their response modality, for example, afferents responsive to mechanical stretch that terminate in the detrusor muscle are sensitive to both contraction and distension, while urothelial afferents are largely insensitive to mechanical stimulation. The mechanosensory properties of lumbar splanchnic and pelvic nerves were characterised in single fibre electrophysiological experiments to distinguish nerves based on their receptive threshold (Xu & Gebhart, 2008; Zagorodnyuk et al., 2007). In a recent study, Christie *et al* (2021) found that mechanosensitivity of each type of bladder afferent was affected by the circadian rhythm, finding the activation thresholds of muscle-urothelial and urothelial afferents significantly higher during the day than the night (Christie et al., 2021). This was supported by Ramsay *et al* (2022) who reported significant attenuation of mechanosensitivity of muscle-urothelial afferents following application of the sleep hormone melatonin (Ramsay et al., 2022). These findings have furthered our understanding of the mechanisms underlying reduced micturition impulses during nighttime, due to the tight regulation of sensory afferent function. The four types of mechanosensitive afferents innervating the bladder are described in detail below, including the muscle, muscle-urothelial, urothelial and serosal afferents (Xu & Gebhart, 2008; Zagorodnyuk et al., 2006, 2007, 2009, 2010).

- Muscle afferents

Most of the afferents terminating in the detrusor layer of the bladder wall are sensitive to distension and are not affected by removal of the urothelium and suburothelium, nor are they responsive to ATP (Zagorodnyuk et al., 2007). This suggests their receptive fields are in the muscle, which explains their response to heavy stroking that distorts the muscle layer but not light stroking (Xu & Gebhart, 2008). Despite this, afferents terminating in the muscle have low activation thresholds to distension. Muscle mechanoreceptors are also considered to be ‘in-series’ tension receptors due to their ability to detect contraction as well as distension, as their terminals lie close to the smooth muscle fibres (Iggo, 1955; Morrison, 1999; Rong et al., 2002). These nerves terminating in the muscle represent 30% of the lumbar splanchnic afferents and 63% of the pelvic afferents (Xu & Gebhart, 2008).

- Muscle-urothelial afferents

The afferent nerves that terminate within the suburothelium and smooth muscle are referred to as ‘muscle urothelial’ afferents. This is due to the finding that removal of the urothelium and lamina propria attenuates the mechanosensitive activity of these nerves (Zagorodnyuk et al., 2007). These afferents have the ability to detect graded increases in distension or stretch, as well as fine stroking, distinguishing them from afferents that terminate only in the detrusor or the urothelium. The advantage of the closeness to the urothelium is shown by the chemosensitivity of muscle-urothelial afferents, as they can detect ATP that may be produced by the urothelium following stretch. Approximately 3% of nerves in the lumbar splanchnic pathway are muscle-urothelial afferents, as well as 14% of the pelvic pathway (Xu & Gebhart, 2008).

- Urothelial afferents

Removal of the urothelial layer greatly diminished the mechanosensitivity of urothelial afferents, leading to the conclusion their terminals lie close to or within the urothelial layer. Urothelial afferents can be divided into two groups, mucosal high responding mechanoreceptors and low responding mechanoreceptors, where high responders were shown to be insensitive to distension or stretch but exhibited robust responses to light stroking (Zagorodnyuk et al., 2007). On the other hand, low-responding afferents were only weakly activated by light stroking and were insensitive to chemical stimulation with αβ-methylene ATP and capsaicin. Urothelial afferents make up 10% of pelvic innervation to the bladder while none have been shown to occur through the splanchnic pathway.

- Serosal afferents

Afferents terminating in the serosa were also insensitive to light stroking, stretch and distension, remaining unresponsive except for blunt probing, an unphysiological stimulus for the bladder (Xu & Gebhart, 2008). Serosal fibres make up the vast majority of afferents travelling along the lumbar splanchnic pathway (67%) and a small percentage of the pelvic pathway (14%) (Xu & Gebhart, 2008). ‘Silent’ afferents such as these have previously been reported to gain the ability to sense mechanical and chemical stimuli following a period of inflammation (Rong et al., 2002; Wen & Morrison, 1995; Xu et al., 2000). Rong et al (2002) found a group of silent fibres that gained mechanosensitivity following application of αβ-methylene ATP, and in a recent study, Brierley et al (2020) found that intravesical application of *E. coli* supernatant, which is responsive for the development of urinary tract infections (UTIs), led to the activation of silent afferents to respond to high intravesical pressures (Brierley et al., 2020; Rong et al., 2002). The quality of silent afferents gaining mechanosensitivity in the presence of pathogenic or inflammatory signals have led to their description as silent nociceptors, where they remain dormant until they must transduce painful signals in pathological situations such as UTI or interstitial cystitis (Brierley et al., 2020; Prato et al., 2017).

Sensory mechanisms within the bladder

Detection and transduction of sensory signals within the bladder is mediated through multi-directional communication between the cell types within the suburothelial layer. The afferent and efferent nerves innervating the bladder are arranged within a suburothelial plexus, in close proximity to urothelial cells, interstitial cells, immune cells and blood vessels. Afferent nerve terminals in the suburothelial layer release neuropeptides such as calcitonin gene related peptide (CGRP), neurokinins, vasoactive intestinal peptide (VIP) and substance P (Bossowska et al., 2015; Sadananda et al., 2008; Zhou et al., 1998). These neuropeptides have been shown to act directly on their neighboring cells, for example, Gillespie (2005) showed that CGRP directly inhibited evoked bladder contractility by activating receptors on the detrusor (Gillespie, 2005). These findings suggest an intricate communication network through which healthy bladder function is maintained.

The terminals of afferent nerves within the suburothelial plexus express a wide variety of receptors, which facilitate detection of mechanical and chemical stimuli. Currently, there are two potential mechanisms by which bladder stretch may be transduced into electrical signals; the direct activation of mechanogated channels, and the stretch induced release of ATP which acts on purinergic receptors on afferent terminals. The identity of the mechanogated channel responsible for transduction of bladder distension is unknown, however potential candidates include the ENaC/ASIC/degenerin channels and transient receptor potential (TRP) channels (see table 1.1 below). Afferent terminals also express a wide variety of sensory receptors which detect the neurotransmitters and neuropeptides released by the various cell types that make up the urothelial associated sensory web (Apodaca et al., 2007).

Receptor	Agonist or stimuli	Localization	Physiological role	References
TRPV1	Heat and protons, capsaicin and resiniferatoxin	DRG and afferent terminals	Nociception and mechanosensation	(Daly et al., 2007; Grundy, Daly, et al., 2018; Hwang et al., 2005; Kobayashi et al., 2005)
TRPV4	Osmolarity and pressure, GSK1016790A	DRG, afferent terminals and urothelium	Osmolarity and pressure sensor	(Aizawa et al., 2012; Ihara et al., 2017; Janssen et al., 2011; Mochizuki et al., 2009; Yoshiyama et al., 2015)
TRPV2	Osmorality, noxious heat >53°C, cannabidiol (CBD)	DRG, urothelium	Mediates CGRP release in the DRG	(Pumroy et al., 2019; N. Qin et al., 2008)
TRPA1	Noxious cold, cinnamaldehyde	DRG and afferent terminals	Mechanosensor and noxious cold sensor	(Andrade et al., 2011; Streng et al., 2008; Zagorodnyuk et al., 2017)
TRPM8	Cool, menthol	Afferents and urothelium	May be implicated in bladder cooling reflex, increased	(Du et al., 2008; Kobayashi et al., 2005; Lashinger et al., 2008; Mukerji et al., 2006)

Receptor	Agonist or stimuli	Localization	Physiological role	References
			expression in idiopathic detrusor overactivity	
P2X2	ATP, α-β methylene ATP	DRG, afferent terminals and urothelium	Enhances afferent firing, nociception	(Cockayne et al., 2005; Munoz et al., 2012; Pannek et al., 2009; Tempest et al., 2004)
P2X3	ATP, α-β methylene ATP	DRG, afferent terminals and urothelium	Enhances afferent firing	(Elneil et al., 2001; Kobayashi et al., 2005; Llewellyn-Smith & Burnstock, 1998; Vlaskovska et al., 2001)
M3	ACh	DRG, afferent terminals and detrusor	Enhances afferent firing	(Chapple et al., 2002; Kullmann et al., 2008)
M2	ACh	C- fibres (spinal cord)	Inhibitory effect on micturition	(Chapple et al., 2002; de Angelis et al., 2014; Masuda et al., 2009)

Receptor	Agonist or stimuli	Localization	Physiological role	References
EP receptors	Prostaglandins	Afferent terminals and urothelium	Increases afferent firing and sensitization through action on NaV1.9 channel	(Lee et al., 2007; Ritter et al., 2009; Su et al., 2008)
Neurokinin receptors	Tachykinins	DRG, afferent terminals and detrusor	NK1 – nociception NK2 – enhances detrusor contraction NK3 – inhibition of micturition reflex	(Grundy et al., 2018; Quinn et al., 2004; Sculptoreanu et al., 2007, 2008; Sculptoreanu & de Groat, 2003; Templeman et al., 2003; Zhang et al., 2007)
TrkA	Nerve growth factor (NGF)	DRG and afferent terminals	Responsible for nerve survival, growth and differentiation. May be involved in the development of chronic pain following bladder injury.	(Fang et al., 2005; Kobayashi et al., 2005; Qiao et al., 2002, 2005)
5-HT3	5-HT	DRG and afferent terminals	Inhibits relaxation of bladder neck through action in the spinal cord, directly modulates	(Chetty et al., 2007; Konthapakdee et al., 2019; Michishita et al., 2015)

Receptor	Agonist or stimuli	Localization	Physiological role	References
			afferent activity through 5HT3 receptor.	
Cannabinoid receptors (CB1 and CB2)	Cannabinoids, endocannabinoids, WIN55,212-2	DRG and afferent terminals, urothelium and detrusor	Decreases afferent activity, reduces electrically stimulated detrusor contraction	(Aizawa et al., 2014; Gratzke et al., 2009; Tyagi et al., 2009; Walczak et al., 2009)
cGMP	Nitric oxide	Afferents, urothelium, interstitial cells	Inhibits afferent firing	(Gillespie et al., 2005a; Okuyama et al., 2021; Smet et al., 1996)
Histamine receptors (H1 and H2)	Histamine	Lumbosacral DRG, urothelial cells, suburothelium and detrusor	H1 - Increases mechanosensitivity through interaction with TRPV1, modulate contractile responses in detrusor H2 – urothelial and suburothelial H2 activation inhibits detrusor contractility	(Grundy et al., 2020; Stromberga et al., 2019)

Receptor	Agonist or stimuli	Localization	Physiological role	References
VPAC1 and VPAC2	PACAP	Afferents, urothelial cells, detrusor	Increase afferent firing, modulate micturition cycle, expression is increased in cystitis.	(Braas et al., 2006; Girard et al., 2016, 2017; Heppner et al., 2019; Ojala et al., 2019)
ENaC	Mechanical stimuli, cold	DRG, afferent terminals and urothelium	Modulates stretch induced ATP release, expression is increased in obstructed bladders.	(Araki et al., 2004; Du et al., 2007)
Acid-sensing ion channels (ASIC)	Low pH and mechanical stimuli	DRG, urothelium, detrusor	Sensitize afferents to extracellular protons, may be involved in nociceptive signalling	(Montalbetti & Carattino, 2021; Yoshiyama et al., 2020)

Table 1.1: A table describing the sensory receptors expressed in the bladder, including the agonist or stimuli recognized by each receptor, where they are expressed and their physiological function. Adapted from de Groat et al (2015).

1.4 Overactive bladder

The International Continence Society defines overactive bladder (OAB) symptomology to be defined by three main symptoms, urgency which may be accompanied by incontinence, frequency and nocturia (see table 1.2; Abrams et al., 2003). A population-based study of OAB prevalence across the UK, Canada, Italy, Sweden and Germany of over 19,000 participants found 11.8% of people reporting OAB symptoms (Eapen et al., 2016). OAB has significant deleterious effects on peoples’ quality of life, with well-established correlations with irritable bowel syndrome (Wyndaele et al., 2011) and psychological disorders such as depression and anxiety (Nicolson et al., 2008). In fact, depression appears to worsen OAB symptoms, with patients reporting lower quality of life than those without depression (Sexton et al., 2011).

The impact on people’s mental health may be worsened by the dearth of treatment options available. There is no cure for OAB, and oral anticholinergic drugs have been shown to have intolerable side effects that makes it difficult for patients to stay on this treatment program. There is also the added complication for elderly patients that anticholinergics may make symptoms of dementia worse (Welk et al., 2020, 2021).

Bladder disorder		**Definition**
OAB	Overactive bladder	A syndrome characterized by urinary urgency (the sudden need to urinate that can’t be deferred to later), frequency (needing to urinate more often) and nocturia (sleep disturbances caused by increased need to urinate) which can be accompanied by incontinence (inability to hold your bladder)
NDO	Neurogenic detrusor overactivity	Involuntary contraction of the smooth muscle of the bladder (the detrusor) during the storage phase. Defined as neurogenic when it occurs due to a neurological condition such as spinal cord injury.
IDO	Idiopathic detrusor overactivity	Involuntary contraction of the detrusor with no known cause.
IC/PBS	Interstitial cystitis/painful bladder syndrome	Pain that accompanies bladder filling, which may lead to frequency and nocturia. For the diagnosis of interstitial cystitis, cystoscopy and histological assessments are necessary to confirm inflammatory state.
BOO	Bladder outlet obstruction	When an obstruction in the urethra (most commonly an enlarged prostate) makes voiding difficult and can increase detrusor pressure due to the increased residual volume in the bladder.

Table 1.2: A table describing bladder disorders and their definitions. Adapted from Abrams et al. (2003).

Current treatments

The first-line treatment of OAB is antimuscarinic (AM) drugs, which have been shown to be relatively ineffective as well as producing intolerable and very common side effects (Kelleher et al., 2018). The newer β3 adrenoreceptor agonists have a better side effect profile but display similar efficacy to the AMs and can be given alongside AMs as combination treatment (Kelleher et al., 2018). A surgical therapy for OAB is sacral neuromodulation, where electrical excitatory and inhibitory stimulation to the spinal S2 nerve root is used to improve continence (Schultz-Lampel et al., 1998; Siegel et al., 2016). As this treatment is very expensive, other treatments must have failed or their side effects intolerable to the patient for it to be funded by the National Health Service (NHS) in the UK (Fletcher, 2020; Park & Chapple, 2019).

The third line treatment for OAB is intravesical (within the bladder) injection of botulinum neurotoxin serotype A (BoNT/A), clinically known as Botox®. BoNT/A injections provide relief from the sensation of urgency and reduce occurrences of frequency episodes, which lasts around six months after injection (Chapple et al., 2013; Dmochowski et al., 2010; Irwin et al., 2013; Karsenty et al., 2014). Side effects include bladder retention, due to the neuroparalytic action of BoNT/A. While the BoNT/A mediated urgency relief are well reproduced in the clinical literature, it remains unclear the mechanism underlying these effects as it preferentially targets and silences cholinergic neurons.

1.5 Botulinum neurotoxins

Botulinum neurotoxins (BoNTs) are a family of potent neurotoxins that target cholinergic nerve terminals and inhibit neurotransmission through the prevention of vesicle docking and fusion. It is the most lethal natural neurotoxin and responsible for causing botulism, a fatal disease characterized by flaccid paralysis and respiratory failure. Despite its lethality, BoNT serotype A (BoNT/A) has been approved for clinical use to treat a variety of disorders, due to its potent neurospecificity, lack of diffusion once injected into the site of interest and the reversibility of its actions. The history, structure, and function of BoNTs are described in detail below.

Botulism

Botulism was first named in the nineteenth century following increased reports of death after consumption of inadequately handled meat. The name botulism comes from the Latin *botulus,* or sausages, and was named by the German doctor Justinus Kerner (1786-1862). Kerner published papers in the 1820s on the autonomic disturbances underlying the “sausage poisoning” he witnessed, his clinical descriptions included muscle paralysis and respiratory distress. Kerner hypothesized that there could be a biological toxin behind the fatal effects, noting that “*the capacity of nerve conduction is interrupted by the toxin in the same way as in an electrical conductor by rust*” (Kerner, 1822). Remarkably, he suggested that clinical utility might be found in the toxin if “*administered in such doses, that its action could be restricted to the sphere of the sympathetic nervous system only, could be of benefit in the many diseases which originate from hyperexcitation of this system*” (Kerner, 1822).

The bacterial culprit behind botulism was discovered in 1897 by the microbiologist van Ermengem and heralded the start of the botulinum neurotoxin (BoNT) research field. After three people died of muscle paralysis induced by consumption of smoked ham, he conducted an autopsy and through examination of the ham, isolated an anaerobic bacterium which he named *Bacillus botulinus* (van Ermengem, 1897). In 1917, reclassification of bacterial nomenclature was conducted to separate the aerobic *Bacillus* genus from the anaerobic *Clostridium* genus, which led to the renaming of *Bacillus botulinus* to *Clostridium botulinum* (Winslow et al., 1917). The environments conducive to growth of *Clostridia* are found in decomposing organic materials such as carcasses, as well as mishandled food including meat and vegetables. Consumption of these materials by vertebrates leads to the transfer of the BoNT across the intestinal wall into the bloodstream, causing flaccid paralysis and death through the targeting and silencing of cholinergic nerve terminals (Rossetto et al., 2014). The potent lethality of BoNTs were uncovered by mouse LD_{50} studies that have shown the fatal dose to be 1 ng BoNT/kg body weight (Gill, 1982), making it one of the most lethal natural neurotoxins in the world. Over the two hundred years following Kerner's identification of foodborne botulism, significant improvements to food preparation methods have rendered it extremely rare, the UK has reported 62 cases since 1922 (McLauchlin et al., 2006). Botulism can also be transmitted through a wound infection, and through intestinal colonization of *C. botulinum* in infants. Wound botulism can be caused by injection of illicit drugs and is more common than foodborne botulism, with an average of 2 reports per year (Brunt et al., 2020).

Genetics

BoNTs are part of a family of neurotoxins classified based on serological methods into seven serotypes, /A to /G, as well as the newly characterized BoNT/FA (Barash & Arnon, 2014; Dover et al., 2014; Hackett et al., 2018), and BoNT/X (Zhang et al., 2017). These serotypes can be subclassified based on differences in the amino acid sequence and named as the original serotype followed by a number for example BoNT/A1, BoNT/A2, BoNT/A3. BoNTs are produced by phylogenetically distinct Clostridial groups, composed predominantly of *C. botulinum*, as well as *C. argentinense, C. baratii* and *C. butyricum* (Rossetto et al., 2014). Different clostridial species can produce the same BoNTs, which may indicate evidence of horizontal gene transfer between the groups (Hill et al., 2015).

The *bont* gene encodes the 150 kDa BoNT proteins, close to the *ntnh* gene which codes for the non-toxic non-haemagglutinin (NTNH) proteins and the *ha* genes responsible for the haemagglutinin (HA) proteins (Hill et al., 2015). The BoNT/A molecule forms a progenitor toxin complex (PTC) with the NTNH and HA proteins, which is stable at acidic pH but dissociates at basic pH. This feature suggests the accessory proteins of the PTC act as a shield to protect the BoNT protein from proteolysis as it travels through the acid and protease rich gastrointestinal tract (Pirazzini et al., 2017). HA proteins do not appear to interact with BoNTs or NTNH proteins, and while their function has not been completely elucidated, it is suspected that they may facilitate the absorption of BoNTs across the mucosal layer of the gastrointestinal tract (Amatsu et al., 2013).

Structure of BoNTs

Although BoNT proteins display some heterogeneity as far as amino acid sequences, their molecular architecture is largely the same. BoNTs are released as a precursor single chain with a molecular mass of approximately 150 kDa, which is activated following cleavage by bacterial proteases to produce a di-chain protein linked by a disulfide bond (Lacy et al., 1998). The resultant neurotoxin is organized into a heavy chain (HC) of 100 kDa, and a 50 kDa light chain (LC), with functionally distinct properties (see figure 1.5). The HC domain contains the receptor targeting domain and the translocation domain, to preferentially target cholinergic neurons and once bound, translocate the LC domain into the cytoplasm. The LC binds to and cleaves its soluble N-ethylmaleimide-sensitive-factor attachment protein receptor (SNARE) target, which are essential for mediating exocytosis, resulting in halted intracellular trafficking and neurotransmitter release (Joussain et al., 2019; Washbourne et al., 1997).

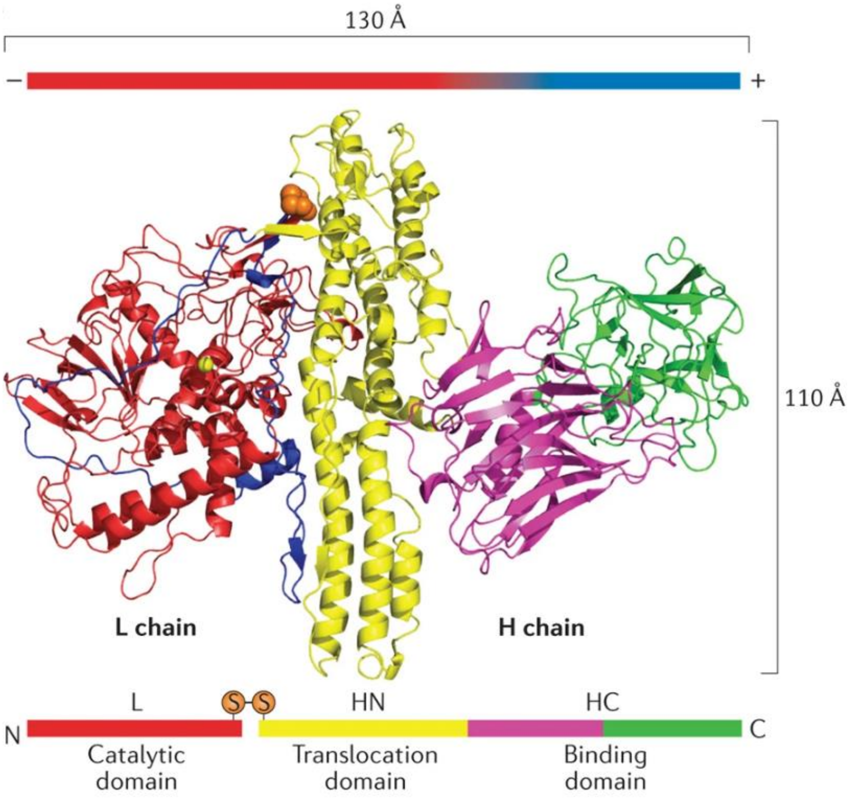


Figure 1.5: The crystal structure of BoNT/A1, the distribution of charges within the molecule as well as the structure and function of its subdomains. The light chain or L chain is the catalytic domain in red, the disulfide bond between the subdomains is pictured as orange S-S. The H chain is composed of the translocation domain (yellow) and the receptor binding domain (purple and green). The 'belt' is pictured in dark blue in the crystal structure and makes a connection between the L chain and the HN domain. Adapted from Rossetto et al. (2014).

Receptor binding and internalization

The specific targeting of cholinergic nerve terminals by BoNTs can be explained by their double-receptor mechanism of entry, due to their interaction with proteins and lipids expressed on specific cell types. The heavy chain of BoNTs bind to synaptic proteins such as synaptotagmins and synaptic vesicle glycoprotein 2 (SV2), as well as lipids such as polysialogangliosides, which are responsible for modulation of membrane proteins and expressed on nerve terminals (Montecucco, 1986). BoNTs /A, /D, /E, /F and /FA bind to SV2, while BoNTs /B and /G have been shown to bind to synaptotagmin I and II (see table 1.3). As SV2 and synaptotagmins are exposed on the surface more commonly in neurons undergoing high levels of exocytosis, binding to these receptors allow BoNTs to silence the most active neurons (Dong et al. 2006). The importance of gangliosides was shown through studies finding that preincubation with gangliosides significantly increased the cellular uptake of BoNT (Schiavo et al., 2000). Following the double receptor binding process, the BoNT molecule is internalized within an endocytic vesicle as it is being recycled. The BoNT molecule takes advantage of the acidification process that occurs within the endocytic vesicle, as it makes a conformational change to insert itself into the membrane and create a channel for the LC domain to pass through (Koriazova & Montal, 2003; Schmid et al., 1993). Once liberated into the cytosol, the LC can find and bind to its SNARE target (see table 1.3).

BoNT serotype	Synaptic vesicle receptor	Ganglioside receptor	SNARE target	References
BoNT/A	SV2C	GT1b and GD1a	SNAP-25 SNAP-23	(Benoit et al., 2013; Blasi et al., 1993; Dong et al., 2006; Vaidyanathan et al., 1999)
BoNT/B	Synaptotagmin I/II	GT1b and GD1a	VAMP	(Dong et al., 2003; Jin et al., 2006; Nishiki et al., 1996)
BoNT/C	Unknown	GT1b, GD1a, GBP2 and Sia-1	Syntaxin and SNAP-25	(Karalewitz et al., 2012; Vaidyanathan et al., 1999)
BoNT/D	SV2	GT1b GD1b GD2	VAMP	(Kroken et al., 2011)
BoNT/E	SV2A and SV2B	GT1b GD1a GQ1b	SNAP-25	(Kamata et al., 1986; Masuyer et al., 2021; Vaidyanathan et al., 1999)

BoNT/F	Sv2	GT1b and GD1a	VAMP	(Fu et al., 2009)
BoNT/G	Synaptotagmin I/II	GT1b	VAMP	(Schmitt et al., 2010)
BoNT/FA	SV2C	Unknown	VAMP	(Yao et al., 2017)
BoNT/X	Unknown	Unknown	SNAP-25 VAMP-1 VAMP-2 VAMP-3 VAMP4 VAMP5 Ykt6 Syntaxin	(Masuyer et al., 2018; S. Zhang et al., 2017)

Table 1.3: A table summarizing the receptor and SNARE targets for BoNTs /A to /X.

Mechanism of neuroparalysis

The mechanism underlying the flaccid paralysis induced by BoNT was first uncovered by Burgen *et al* (1949), who conducted a rat hemidiaphragm preparation to show electrically stimulated contraction was inhibited following exposure to BoNT/A (Burgen et al., 1949). In this influential study, the investigators also directly measured ACh release and found that the paralysed NMJ released significantly less ACh than untreated NMJs (Burgen et al., 1949). This method has been used widely in the literature to characterize BoNTs, uncovering mechanisms of entry and intracellular activities (Dolly et al., 1987; Elliott et al., 2017; Rasetti-Escargueil et al., 2009; Rummel et al., 2007; Simpson, 1973, 1974).

The process by which clostridial neurotoxins inhibit neurotransmitter release and the subsequent muscle contractility was characterized in the early 90s, as Schiavo *et al* (1992) found both tetanus toxin and BoNT/B cleaved the SNARE protein synaptobrevin, or VAMP (Schiavo et al., 1992). Later, Blasi *et al* (1993) showed that BoNT/A targeted and cleaved SNAP-25, another SNARE (Blasi et al., 1993). SNARE proteins are essential for the maintenance of intracellular trafficking, they are differentiated into v-SNAREs and t-SNAREs depending on their placement on the vesicle and plasma membrane respectively, and drive membrane fusion by wrapping around each other and pulling the vesicle to the membrane to release its neurotransmitter contents (Daste et al., 2015). The consequence of BoNT induced SNARE cleavage is impairment of intracellular trafficking mechanisms, which affects multiple cellular functions including communication through neurotransmitter release and receptor expression (Gundersen, 1980; Ray et al., 1993; Xiao et al., 2011). A few BoNTs share cleavage targets, for example both BoNTs /A and /E cleave SNAP-25, however at different points which affects the extent of their neuroparalysis, as BoNT/E intoxication is markedly shorter both *in vivo* and *in vitro* (Beske et al., 2017; Binz et al., 1994; Eleopra et al., 1998).

Clinical formulations

Although multiple serotypes of BoNT have been discovered, the most commonly used in the clinic for aesthetic and therapeutic indications is BoNT/A1. The approved formulations of BoNT/A1 are produced using the Hall strain of *Clostridium botulinum* and are split into onabotulinum toxin A (Botox ®; Allergan, USA), abobotulinum toxin A (Dysport ®; Ipsen, France) and incobotulinum toxin A (Xeomin ®; Merz, Germany) (Field et al., 2018). The main differences in the formulations lie in the manufacturing and development, as Botox and Dysport contain the entire progenitor toxin complexes (PTCs) including NTNH and HA proteins, whereas Xeomin is composed solely of the 150 kDa BoNT/A1 protein (Pirazzini et al., 2017). The potency of each product is defined by the mouse lethality bioassay and expressed in units (U), where the LD_{50} corresponds to 1U. The variations in composition of the BoNT/A products may explain the differences in clinical efficacy, as studies have shown dose conversion ratios of Botox and Dysport to be 1:2 while Botox and Xeomin were between 0.5:1 and 0.75:1 (Kutschenko et al., 2016). This influences the recommended dosages made by regulatory agencies such as the FDA, for example in adult upper limb spasticity, the recommended doses are 1000U Dysport, 400U Botox and 400U Xeomin (Field et al., 2018).

BoNT/B has also been approved by the FDA as rimabotulinum toxin B (Myobloc ®, Solstice Neurosciences, USA), and is approved for use in certain indications such as cervical dystonia, however the dosages are much higher (around 2500U to 5000U) possibly due to its decreased potency due to its lower affinity for the human synaptotagmin protein (Spiegel et al., 2020; Tao et al., 2017).

1.6 The effect of BoNT/A on bladder function

Clinical use of BoNT/A in bladder disorders

BoNT/A is administered routinely as a third line treatment for OAB across the world, as it has shown efficacy in the reduction of OAB symptomology and improving quality of life for patients. The first study using BoNT/A in the bladder was conducted by Dykstra et al (1988) who injected BoNT/A into urethral sphincters of patients with spinal cord injury and detrusor-sphincter dyssynergia, finding that the majority of the patients had improvements in urinary retention (Dykstra et al., 1988). Over the years, other potential urological applications were tested until BoNT/A was approved for use in the US for OAB in patients not responsive or intolerant to anti-cholinergic drugs (Federal Drug Administration, 2013).

Schurch et al (2000) were the first to inject BoNT/A directly into the bladder wall, by using a cystoscope to enter the bladder through the urethra (Schurch et al., 2000). Since the turn of the millennium, there have now been several clinical trials studying the effectiveness of BoNT/A in treating OAB using protocols that vary in total dose, areas of injection and numbers of injections (Chapple et al., 2013; Denys et al., 2012; Dmochowski et al., 2010; Ginsberg et al., 2012; Kuo et al., 2016; Schurch et al., 2005). The earlier studies injected up to 300U of BoNT/A into the bladder wall but found a dose dependent increase in adverse effects such as urinary retention (Dmochowski et al., 2010). Current UK National Institute for Health and Care Excellence (NICE) guidance now recommends the dose of 100U injections of BoNT/A in the bladders of women with OAB, increasing the dose to 200U if symptoms persist.

Surprisingly despite its clinical use, the mechanism which underlies the effectiveness of BoNT/A for the treatment of lower urinary tract (LUT) disorders has not been fully described. BoNT/A could be working via a classical mechanism involving the paralysis of the bladder smooth muscle by silencing cholinergic nerves through cleavage of synaptosomal-associated protein 25 (SNAP-25;

Blasi et al. 1993). The appearance of urinary retention in those treated with BoNT/A certainly lends weight to this idea. However, many clinical trials have shown significant reductions in the sensation of urgency in OAB patients (Chapple et al., 2013; Cruz et al., 2011; Dmochowski et al., 2010) and pain in interstitial cystitis/painful bladder syndrome (IC/PBS) patients (Chung et al., 2012; Kuo & Chancellor, 2009). BoNT/A treatment significantly improves patients' quality of life (Pinto et al., 2010), possibly due to the amelioration of sensory symptoms as boosts in quality-of-life scores were found to be significantly correlated with reductions in episodes of urgency and incontinence (Kalsi et al., 2006). This has led to the suggestion that BoNT/A could also be working by a secondary non-classical mechanism to inhibit sensory neurotransmission.

As patients with OAB and IC/PBS report loss of urgency and pain associated with bladder filling after BoNT/A treatment, there is the possibility that BoNT/A's effects are most prominent in the storage phase of micturition (Chapple et al., 2013; Kuo et al., 2016; Smith et al., 2004). As the bladder fills, it receives sympathetic stimulation to relax the detrusor smooth muscle and maintain low pressures to allow increases in volume. The parasympathetic cholinergic stimulation only occurring when the detrusor contracts to empty. As previously discussed, the storage phase is not controlled by parasympathetic pathways, and the cardinal symptoms of OAB and IC/PBS are sensory symptoms (i.e., urgency or pain) and occur during urine storage. Therefore, it is unlikely that BoNT/As effect on urgency and /or pain is due to inhibition of cholinergic neurotransmission. Interestingly, Silva et al (2000, 2007) instilled resiniferotoxin, a TRPV1 specific toxin into bladders of patients with OAB and showed that inhibition of sensory pathways reduces the sensation of urgency (Silva et al., 2000, 2007). This suggests that the development of urgency could depend on dysregulation of afferent pathways, and to reduce these symptoms BoNT/A could work at least partly by modulating these pathways.

Expression of BoNT/A's receptor and target protein in the bladder

Previous studies have reported significantly reduced plasma membrane expression of sensory receptors following BoNT/A treatment (Apostolidis et al., 2005; Ha et al., 2011; Liu et al., 2015; Xiao et al., 2011). Many groups have also shown inhibition of urothelial ATP release post-BoNT/A application (Collins et al., 2013; Hanna-Mitchell et al., 2015; Khera et al., 2004; Smith et al., 2005, 2008). While these findings have been reproduced extensively, the mechanism that underlies these processes have not been elucidated.

The presence of SNAP-25 in urothelial cells has been disputed (see table 1.4). Hanna-Mitchell et al (2015) showed presence of SNAP-25 in both human and rat urothelial cells which was significantly reduced following BoNT/A application (Hanna-Mitchell et al., 2015). Other studies conducted by El-Shatoury et al (2018) using rat bladders and Kuo et al (2014) using human tissue from OAB biopsies also show evidence to support SNAP-25's presence in the urothelium (Kuo et al., 2014; El-Shatoury et al., 2018). Interestingly, Born et al (2003) investigated the expression of SNARE proteins in the rat urothelium and did not find SNAP-25, instead finding that the SNARE complex in the rat urothelium is composed of SNAP-23, synaptobrevin and syntaxin (Born et al., 2003).

Most other studies that have shown evidence of SNAP-25 presence in the bladder have concluded it is restricted to the nerve fibres innervating the suburothelium and muscle layers (Chuang et al., 2009; Coelho et al., 2010, 2012; Liu et al., 2015; Oliveira et al., 2017; Schulte-Baukloh et al., 2007). Coelho et al (2012) showed by double-labelling cleaved SNAP-25 (cSNAP-25) with vesicular acetylcholine transporter (VAChT), tyrosine hydroxylase (TH) and calcitonin gene-related peptide (CGRP) that 85% of VAChT positive neurons expressed cSNAP-25, whereas only 42% and 36% of neurons were TH positive and CGRP positive respectively (Coelho et al., 2012). Interestingly, in the same study Coelho et al (2012) intravesically instilled the toxin and found no cSNAP-25-IR in the suburothelial nerve fibres, although reduced afferent signalling without injection has been reported by multiple groups (Chuang et al., 2004; Collins et al., 2013; Lucioni et al., 2008; Rapp et al., 2006). The incongruity in these findings may be attributable to different nerve populations giving different results, or that instillation did not allow proper penetrance of BoNT/A. The mechanisms of BoNT/A entry and action and their importance to the modulation of bladder mechanosensitivity was further investigated in chapters three and five of this thesis.

Author	Species	Where is SV2 expressed?	Where is SNAP-25 expressed?	Method	Findings
(Hanna-Mitchell et al., 2015)	Rat and human	Urothelium	Urothelium	Immunohistochemistry and gel electrophoresis of rat and human bladder mucosa (urothelium and lamina propria) and cultured urothelial cells	BoNT/A significantly reduced SNAP-25 expression in both rat and human. Rat tissue has more SNAP-23 than SNAP-25
(Coelho et al. 2010)	Human	Nerve fibres in the suburothelium and detrusor	Nerve fibres in the suburothelium and detrusor	Human bladders from organ donors were used for immunofluorescence staining of SV2 and SNAP-25	Dense SV2 and SNAP-25 immunoreactivity in the suburothelium and detrusor layer, in fibres also positive for neuronal marker β-3 tubulin. They found no labelling within urothelium or muscle cells. SV2-IR colocalised with VAChT and CGRP positive fibres
(Kuo et al., 2014)	Human	Urothelium and suburothelial fibres	Urothelium and suburothelial fibres	Instilled 200U Lipotoxin (liposome encapsulated BoNT/A) into bladders of OAB patients, IHC for SV2 and SNAP-25 expression before treatment and 3 months after	IHC and western blotting shows SV2 expression in the urothelium (apical cells and suburothelium). SNAP-25 expression in the urothelium and suburothelial nerve fibres, decreased post-treatment in patients responding to treatment.

(Liu et al., 2015)	Human	Bladder mucosa (including the urothelium, lamina propria and a few discontinuous muscularis mucosa)	Bladder mucosa (including the urothelium, lamina propria and a few discontinuous muscularis mucosa)	Injected BoNT/A and instilled Lipotoxin intravesically in bladders of OAB patients. Collected samples at baseline before treatment and 1 month after. Conducted IHC on bladder biopsies and immunoblotting on urothelium samples	Patients who responded to BoNT/A injection exhibited a significant decrease in SNAP-25 expression but lipotoxin responders did not. Lipotoxin responders however showed a significant decrease in P2X3 expression.
(Schulte-Baukloh et al., 2007)	Human	*did not look for SV2	Intradetrusor nerve fibres	Patients with NDO received intradetrusor BoNT/A. western blot and immunofluorescence for SNAP-25 and cleaved SNAP-25 expression	Detected SNAP-25 and cleaved form (SNAP-25$_{A}$) in detrusor using western blotting and IF
(Coelho et al., 2012)	Guinea pig	*did not look for SV2	Suburothelial nerve fibres only	Guinea pig bladders were treated with BoNT/A, through intravesical instillation and intramural injection, stained bladders for cleaved SNAP-25	Cleaved SNAP-25 immunoreactive fibres in the mucosa and muscular layer. BoNT/A instillation did not cleave SNAP-25
(El-Shatoury et al., 2018)	Rat	*did not look for SV2	Urothelium, suburothelium and detrusor	Bladders were instilled with 10U BoNT/A alone, injected into detrusor, BoNT/A with an Alexa Fluor tag attached, or BoNT/A with HA. Performed IHC and IF	Injected BoNT/A showed very little SNAP-25-IR, being more effective at cleaving it. While BoNT/A instillation showed dense SNAP-25 IR in the urothelium
(Chuang et al., 2009)	Rat	*did not look for SV2	Suburothelial nerve fibres	Instilled bladders with liposomes, BoNT/A or lipotoxin,	BoNT/A instillation showed significant staining of

				8 days later animals sacrificed and bladders fixed. Performed IF and western blots for SNAP-25 expression.	SNAP-25 in nerve fibres, more than lipotoxin, which suggests the lipotoxin is better at cleaving SNAP-25. Western blots also show significantly less SNAP-25 following lipotoxin
(Born et al., 2003)	Rat	*did not look for SV2	No expression in the urothelium	Conducted IF, electron microscopy and western blots on the urothelium to look for expression of SNARE proteins	SNAP-25 was not found in the urothelium, through IF or westerns, however was present in choroid plexus (brain stem) tissue that was used as control. Showed that SNARE complex in the urothelium is made of SNAP-23, synaptobrevin and syntaxin
(Oliveira et al., 2017)	Mouse	*did not look for SV2	Suburothelial nerve fibres	Injected 0.5U of Botox or Dysport into dome of mouse bladders, three days later conducted IHC of cleaved SNAP-25	injection of both forms of BoNT/A led to cleavage of SNAP-25 in nerve fibres of the lamina propria. However, images show the whole bladder, and zoom in only on the lamina propria. Appears to be some staining in the urothelium of the Botox treated bladder
(Wankel et al., 2016)	Mouse	*did not look for SV2	No expression in the urothelium	Conducted immunoblotting to compare expression of v-SNAREs and t-SNAREs in the urothelium and bladder	Showed that the mouse urothelium expresses SNAP-23 but not SNAP-25

(Yiangou et al., 2011)	Human	Dorsal root ganglion neurons and throughout the bladder	*did not look for SNAP-25	Collected bladder tissue from patients with IDO, PBS and controls, cultured human DRG neurons Conducted IHC on bladder tissue and calcium imaging on DRG neurons.	SV2 was expressed in DRG neurons, immunoreactivity significantly increased in injured neurons. SV2 expressed in bladder; detrusor muscle, blood vessels, urothelium and suburothelium, expression significantly higher in patients with IDO and PBS.
(Giannantoni et al., 2011)	Human	Urothelial cells	*did not look for SNAP-25	Cultured human urothelial cell lines and conducted PCR	Urothelial cells express SV2-A and SV2-B
(Ray et al., 2003)	Human	Parasympathetic nerves innervating detrusor	*did not look for SNAP-25	Biopsies of detrusor muscle from patients with sensory urgency and control patients, conducted IHC for SV2 and P2X receptors	Used SV2 as a neuronal marker for parasympathetic nerves. There was no staining in detrusor muscle itself.

Table 1.4: A table summarising the studies that have investigated expression of SNAP-25 and SV2 in the bladder, including the author, species, cell type, methods and results obtained from each study. Adapted from Ibrahim et al (2022).

Entry of BoNT/A into cells of interest

The urothelium is one of the most impermeable epithelial layers in the body, specialised to maintain a barrier between the blood and waste products in the urine through tight junctions and apical membranes (Khandelwal et al., 2010). Multiple studies have intravesically instilled BoNT/A and found direct action on SNARE dependent and independent release of neurotransmitters and mechanosensitivity of afferent nerves, suggesting BoNT/A is able to traverse the impermeable barrier somehow (Chuang et al., 2004; Collins et al., 2013; Lucioni et al., 2008; Rapp et al., 2006). There are multiple potential ways BoNT/A may interact with the urothelium, it may enter the urothelium directly and cleave SNAP-25 to inhibit exocytosis. It may also move through the cell through transcytosis or move through the tight junctions between urothelial cells to reach suburothelial structures such as afferent nerves and interstitial cells.

Although the expression of SV2 in the urothelium is disputed, Jacky et al (2013) identified fibroblast growth factor receptor 3 (FGFR3) as a receptor for BoNT/A, finding that higher FGFR3 expression in neuronal cells led to increased toxin internalization (Jacky et al., 2013). Bomba-Warczak et al (2016) also reported increased cleavage of SNAP-25 in neurons after expression of FGFR3 (Bomba-Warczak et al., 2016). This receptor could potentially facilitate BoNT/A uptake in the urothelium, as FGFR3 expression has been shown in the urothelium (Akanksha & Sandhya, 2019).

During bladder filling, the umbrella cells undergo exocytosis to release uroplakin-containing fusiform vesicles to increase the surface area and accommodate the stretch caused by increasing volumes. When the bladder contracts to empty, the process of compensatory endocytosis is initiated to recover that extra membrane (Khandelwal et al., 2010). The mechanism underlying the exocytosis of the fusiform vesicles is a systematic process controlled by Rab GTPases which regulate vesicular release through SNARE proteins (Born et al., 2003; Khandelwal et al., 2008; Wankel et al., 2016). Wang et al (2003) described the effect of mechanical stretch on the surface area of umbrella cells, using modified Ussing chambers they applied hydrostatic pressure on the urothelium and found that transepithelial capacitance, a measure of surface area, increased as pressure increased (Wang et al., 2003). This was further expanded on by Yu et al (2009) who showed that it was not necessarily pressure itself, but the increased stretch applied to the membrane and concomitant Ca^{2+} influx that initiated the release of fusiform vesicles (Yu et al., 2009). If BoNT/A was able to enter the umbrella cells through receptor-mediated endocytosis, it follows that it may cleave SNAP-25 (or SNAP-23 at higher concentrations) and interrupt endocytic vesicle recycling in umbrella cells. This may have a significant effect on urothelial mechanosensation, as rises in membrane tension directly leads to increased release of ATP (Lewis & Lewis, 2006). This hypothesis has not been tested previously and if tested, could provide another mechanism by which BoNT/A alters bladder function.

Due to numerous studies finding that BoNT/A affects the activity of cells that lie within the suburothelium, it may be possible that BoNT/A travels further upon entering the urothelial cells. This may occur through the process of transcytosis, BoNT/A entering the cell through an endocytic vesicle and being transported to the basal side to be released. Transcytosis of BoNT/A across cellular layers has been well described in different physiological systems, including epithelial cells of the colon and lungs, as well as neurons (Ahsan et al., 2005; Al-Saleem et al., 2012; Bomba-Warczak et al., 2016; Couesnon et al., 2007; Ghosal et al., 2018; Maksymowych & Simpson, 2004; Park & Simpson, 2003; Restani et al., 2011). In the intestine, BoNT/A has been shown to cross the epithelial barriers, in the apical to basolateral direction (Ahsan et al., 2005). Couesnon *et al* (2007) conducted a study on the passage of BoNT/A and its associated proteins through cultured intestinal crypt cells, finding that transcytosis depended on receptor binding as it was facilitated by the heavy chain (Couesnon et al., 2007). Further studies on *ex vivo* preparations of mouse ileum where the BoNTA was administered intraluminally into the segment showed that spontaneous and electrical field stimulation-evoked muscle contraction was significantly inhibited, leading to the conclusion that BoNT/A may have travelled through the intestinal layers to reach cholinergic nerve terminals (Couesnon et al., 2012). Previous research has shown this mechanism of transcytosis is mediated by the complexing proteins NTNH/A and the haemagglutinins (Ghosal et al., 2018).

Effect of BoNT/A on urothelial mechanosensation

Studies into mechanosensation in primary urothelial cells have shown that mechanical stretch leads to an influx of calcium ions which triggers ATP release (Mochizuki et al., 2009). Mochizuki et al (2009) showed that subjecting these cells to Ca^{2+} free media significantly attenuated stretch-induced intracellular Ca^{2+} increase, as well as attenuating stretch-induced ATP release (Mochizuki et al., 2009).

The importance of intracellular Ca^{2+} influxes to urothelial mechanosensation raises interesting questions as to what impact BoNT/A could have on these processes, as SNAP-25 is vital for the maintenance of intracellular Ca^{2+}dynamics (Verderio et al., 2004). Alderton et al (2000) showed that BoNT/A led to inhibition of store operated Ca^{2+} (SOC) entry in a human kidney cell line, as well as other vesicular release inhibitors tetanus neurotoxin (TeNT) and brefeldin A, concluding that vesicular fusion may modulate the opening of SOC channels (Alderton et al., 2000). Taking these findings together with those of Matsumoto-Miyai et al (2011), the potential effect of BoNT/A on urothelial SOC channels could be that the lack of fine tuning could alter urothelial release mechanisms, and as the SOC channels are meant to replenish ER stores, over time this could lead to a global reduction in stretch-induced ATP release. However, this has not been shown experimentally. Interestingly, BoNT/A intoxication of cells is quite sensitive to Ca^{2+} dynamics, multiple groups have shown that BoNT/A-induced blockade of vesicular release can be rescued by increasing extracellular Ca^{2+} concentrations (Beske et al., 2017; Capogna et al., 1997; Sakaba et al., 2005). Sakaba et al (2005) showed that BoNT/A reduced the Ca^{2+} sensitivity of cells and moved the dose response curve to the right but did not change its shape (Sakaba et al., 2005).

Voltage gated Ca^{2+} channels (VGCCs) have also been shown to interact with SNAP-25 and syntaxin-1 (Rettig, 1996). These findings suggest potential consequences of SNARE cleavage on Ca^{2+} dynamics which could affect mechanosensation and urothelial function more broadly.

Effect of BoNT/A on afferent signalling

The finding that BoNT/A treatment inhibits afferent signalling and neuropeptide release has been shown in different experimental conditions and nerve types. Rapp et al (2006) showed BoNT/A inhibited ATP and capsaicin induced CGRP release from rat bladder (Rapp et al., 2006). Lucioni et al (2008) conducted a study on the effect of BoNT/A on acute (hydrochloric acid application) and chronic inflammatory pain (cyclophosphamide injection) on whole rat bladder tissue (Lucioni et al., 2008). They showed that BoNT/A significantly reduced the release of both substance P and CGRP, to halt the development of neurogenic inflammation and resulting pain (Lucioni et al., 2008). These findings suggest direct action of BoNT/A on bladder signalling that may be attributed to the well-described mechanism of SNAP-25 cleavage.

An example of BoNT/A effects on sensory signalling that may not so easily be explained by SNAP-25 cleavage is its direct inhibition of afferent excitability. Burstein et al (2014) conducted a study on the effect of BoNT/A application on mechanosensitivity of meningeal afferent nerves, by recording the conduction velocities of the single units they were able to identify C- and Aδ fibres (Burstein et al., 2014). By applying von Frey filaments with increasing force to the receptive fields of those nerves, they were able to show increased afferent discharge in response. BoNT/A reduced the firing of C-fibres but not Aδ-fibres, which is interesting as C-fibres are considered to transduce high threshold or painful signals, although other studies have found no correlation between conduction velocity and activation threshold (Sengupta & Gebhart, 1994; Shea et al., 2000; Su et al., 1997).

In the Collins et al (2013) study, they performed single unit analysis post-hoc using the Spike2 software to identify single units and attribute them to the point at which they started firing (previously described by Rong et al., (2002)). Collins et al (2013) found that BoNT/A inhibited responses from both low threshold (69% decrease) and high threshold (65% decrease) afferents in response to distension. However, some caution should be taken when interpreting these data as spike sorting is an estimation of C- and Aδ-fibres, as the nerve fibres they recorded from are multi-unit.

These studies describe direct action of BoNT/A on afferent excitability and ability to transduce mechanical signals, which cannot be easily rationalised by its well-described inhibition of exocytotic mechanisms. While many studies have proposed SNAP-25 independent actions of BoNT/A, there has been little concrete evidence that supports this hypothesis. In chapter five, the SNAP-25 independent functions of BoNT/A were investigated by testing the activity of a catalytically inactive construct of BoNT/A.

1.7 Research aims

The main aim of this thesis was to further characterize the function of BoNTs in the bladder, to better understand the effects on sensory signalling and transduction of mechanical signals.

The direct action of BoNT/A on sensory nerve excitability has been described previously, in multiple organ systems (Collins et al. 2013; Burstein et al. 2014). The mechanisms underlying this effect was not clear, especially as there was no consensus in the literature as to the expression of the receptor and cleavage targets of BoNT/A. The main aim of this project was to study this phenomenon in depth, using a variety of native and recombinant as well as catalytically active and inactive BoNTs to better understand how these neurotoxins may inhibit sensory signalling. The research questions of this project were the following;

- Is the entry of BoNT/A dependent on the double receptor mechanism of SV2 and ganglioside binding?

- Does BoNT/A cleave SNAP-25 to modulate bladder mechanosensation? What happens if a catalytically inactive version of BoNT/A is used?

- What effect do other BoNT serotypes such as BoNT/B and BoNT/E have on bladder mechanosensation?

These questions were tackled using an *ex vivo* multi-unit bladder electrophysiology assay, where intravesical pressure and afferent nerve responses to distension were recorded concomitantly. Immunohistochemistry was conducted using antibodies targeting both the full-length and cleaved SNAP-25 in bladders pretreated with BoNT/A, to confirm the expression of SNAP-25 in the bladder and its location, as well as investigating the contribution of SNAP-25 cleavage to the effect of BoNT/A on bladder mechanosensitivity

CHAPTER 2 – MATERIALS AND METHODS

2.1 Botulinum neurotoxins (BoNTs)

Research grade natural BoNTs /A, /B and /E were purchased from List Biological Laboratories (Campbell, USA), Dysport was provided by Ipsen (Milton Park, UK). Recombinant forms of BoNT/A were produced as described below by Ipsen employees.

BoNT/A1 gene synthesis as performed by IPSEN employees

The BoNT/A1 protein sequence (UPI0000001386) was back-translated and codon-optimised for expression in *E. coli* (DNA 2.0, Menlo Park, CA, USA). The DNA sequence was synthesised in two parts, for safety, and subsequently combined, at a silent KpnI restriction site, to create a coding sequence for the full-length neurotoxin. Silent 5' NdeI and 3' HindIII sites were incorporated and used to insert the open reading frame into a pJ401 expression plasmid (DNA2.0) to create pJ401-BoNT/A1.

Amino acid numbers of the protein sequences cited are counted from the predicted sequence of the encoded protein, including the initiating methionine.

Expression and purification of BoNT/A1 as performed by IPSEN employees

All manipulations of material containing full-length neurotoxin were performed in microbiological safety cabinets, located in restricted-access Containment Level II laboratories. pJ401-BoNT/A1 was transformed into BLR (DE3) cells and expressed as 1L cultures, grown in animal component-free culture medium (AF, 12 g/L phytone peptone animal free, 24 g/L yeast extract, 10 g/L glycerol, 76 mM potassium phosphate dibasic and 14 mM potassium phosphate monobasic, 0.2% glucosamine, 30 µg/ml kanamycin), in 2.5 L shake flasks. Expression cultures were inoculated with a 1:100 dilution from an overnight starter culture and incubated at 37 °C with shaking until OD_{600} 0.5-0.6 was reached. The temperature was reduced to 16 °C and the cultures equilibrated for 1 h, then induced with IPTG (final concentration of 1 mM) and grown for a further 20 h.

Cells were harvested by centrifugation (4300 g, 10 min) and the cell-pellets stored at -20 ^{o}C. Cell pellets were thawed at room temperature and resuspended (3 ml/g) in lysis buffer (35 mM NaCl in 50 mM Tris pH 8.0) supplemented with 10 µl of Benzonase. Cells were lysed by ultrasonication at 4 °C (Misonix 3000 sonicator, 1 cm diameter probe, 10 x 30 second pulses, power setting 4.5, output 60-80 W). The resultant lysate was clarified by centrifugation (4300 g, 1 h, 4 °C) and the supernatant retained. This supernatant was adjusted to 17.5 mM NaCl, 1.1 M $(NH_4)_2SO_4$, 50 mM Tris pH 8.0, by adding an equal volume of 2.2 M $(NH_4)_2SO_4$ in 50 mM Tris pH 8.0; centrifuged (4300 g, 60 min, 4 °C), and loaded (4 ml/min flow rate) onto three stacked 5 ml HiTrap™ Butyl HP columns (pre-equilibrated with 1.1 M $(NH_4)_2SO_4$ in 50 mM Tris pH 8.0). Columns were washed with 15 column volumes (CV) of 1.1 M $(NH_4)_2SO_4$ in 50 mM Tris pH 8.0, then eluted over a 20 CV linear gradient (1.1 to 0 M $(NH_4)_2SO_4$ in 50 mM Tris pH 8.0) and collected as 10 ml fractions.

Fractions were monitored by SDS-PAGE (NuPAGE™ 4-12% Bis-Tris gels) stained for total protein (SimplyBlue™ SafeStain) and those containing target protein were pooled and stored at 4 °C overnight. Pooled material was desalted (HiPrep 26/10 Desalting column) into 50 mM Tris pH 8.0 as a series of 11 ml batches. Each batch was collected until the conductivity surpassed 3.0 mS/cm, at which point the column was re-equilibrated until the conductivity dropped back below 3.0 mS/cm for the next batch. This process was repeated until the entire pooled fraction had been buffer exchanged. The desalted sample was loaded at 5 ml/min onto a 5 ml itrap™ Q HP column (previously washed and charged following manufacturer's guidelines and pre-equilibrated with 50 mM Tris pH 8.0). The column was washed with 15 CV (50 mM Tris pH 8.0), and eluted at 5 ml/min, 15 CV linear gradient (0 to 300 mM NaCl in 50 mM Tris pH 8.0), with 2.5 ml fractions collected. Fractions were monitored by SDS-PAGE (NuPAGE™ 4-12% Bis-Tris gels) stained for total protein (SimplyBlue™ SafeStain) and those containing target protein were pooled and stored overnight at 4 °C.

Protein concentration was determined by Bradford assay and the sample was concentrated to 9 ml in a Vivaspin 20 centrifugal concentrator (MwCO 5000 Da, 4300 g, 20 min cycles at 4°C), protein concentration was measured again by Bradford assay and adjusted to 0.5 mg/ml with 125 mM NaCl in 50 mM Tris pH 8.0. Endoproteinase Lys-C was added (final Lys-C concentration 0.8 µg/ml) and the sample incubated for 20 h at 4 °C. The sample was adjusted with an equal volume of 2 M $(NH_4)_2SO_4$, 50 mM Tris pH 8.0 and loaded onto two stacked 1 ml HiTrap™ Phenyl HP columns at 1.0 ml/min (pre-equilibrated with 1 M $(NH_4)_2SO_4$ in 50 mM Tris pH 8.0).

The column was washed with 10 CV of the same buffer and eluted with a 15 CV linear gradient (1 - 0 M $(NH_4)_2SO_4$, 50 mM Tris pH 8.0) at 2 ml/min, collected as 1 ml fractions. Fractions were monitored by SDS-PAGE (NuPAGE™ 4-12% Bis-Tris gels) stained for total protein (SimplyBlue™ SafeStain) and those containing target protein were pooled, concentrated and diafiltered into phosphate buffered saline (KH_2PO_4 1 mM, Na_2HPO_4 3 mM, NaCl 155 mM, pH 7.4) (Vivaspin 20 centrifugal concentrator MwCO 5000 Da, 4300 g, 20 min cycles at 4°C), concentration was again determined by Bradford assay and adjusted to 0.1 mg/ml before storage as aliquots at -80 ^{o}C.

Structures of BoNTs

The details of BoNTs and subdomains used in this study are described in table 2.1, including the structure, receptor and catalytic targets, method of production and concentrations used.

Name of BoNT	Description	Receptor target	Catalytic target	Production method	Concentrations used
Dysport	BoNT/A complex (>500 kDa)	SV2	SNAP-25	Purified from *C.botulinum*	100U/ml or 3.6 pM
nBoNT/A	BoNT/A only (150 kDa)	SV2	SNAP-25	Purified from *C. botulinum*	3.6pM, 300 pM
rBoNT/A	BoNT/A only (150 kDa)	SV2	SNAP-25	Recombinant; expressed in *E.coli* and purified	3.6pM, 300 pM
rLHn/A	BoNT/A without HCc domain	No targeting domain	SNAP-25	Recombinant; expressed in *E.coli* and purified	1fM, 3.6pM, 300 nM, 1µM
rLC/A	Light chain only	No targeting domain	SNAP-25	Recombinant; expressed in *E.coli* and purified	3.6pM
rHC/A	Heavy chain only	SV2	No catalytic domain	Recombinant; expressed in *E.coli* and purified	3.6pM

Table 2.1: BoNT formulations and constructs used in this thesis, continued also on the next page. The receptor and catalytic target of each BoNT or construct is described as well as method of production, size of the protein or protein complex and the concentrations used. SV2 – synaptic vesicle 2; SNAP-25 – synaptosomal associated protein of 25kDa. The ‘r’ and ‘n’ prefixes (for example rBoNT/A or nBoNT/A) are used to distinguish between recombinant (r) and natural (n) versions of the neurotoxin.

Name of BoNT	Description	Receptor target	Catalytic target	Production method	Concentrations used
rBoNT/A (0)	BoNT/A with mutated light chain	SV2	Catalytically inactive	Recombinant; expressed in *E.coli* and purified	1fM, 3.6pM
rLC/A (0)	Mutated light chain only	No targeting domain	Catalytically inactive	Recombinant; expressed in *E.coli* and purified	3.6pM
rTrkA TSI	BoNT/A with heavy chain removed and TrkA targeting antibody attached	TrkA	SNAP-25	Recombinant; expressed in *E.coli* and purified	3.6pM
nBoNT/B	BoNT/B only	Synaptotagmin I and II	Synaptobrevin	Purified from *C. botulinum*	3.6pM
nBoNT/E	BoNT/E only	SV2	SNAP-25	Purified from *C. botulinum*	3.6pM

Table 2.1 continued from previous page

2.2 Animals

These studies were performed using adult C57BL/6J mice between 8 and 12 weeks old (Charles River Laboratories, Margate, Kent). On the day of the experiment, mice were delivered and sacrificed using a rising concentration of CO_2 in accordance with Schedule 1 of the Animals (Scientific Procedures) Act 1986. Experiments performed were in accordance with ethical approval obtained from the UCLan Animal Welfare and Ethics Review Board (AWERB) (reference RE/16/11).

2.3 *Ex vivo* bladder electrophysiology

The *ex* vivo bladder electrophysiology preparation was conducted as previously described (Daly et al., 2007; 2014; Rong et al., 2002; Vlaskovska et al., 2001; Grundy et al., 2018; 2019; 2021). Following CO_2 asphyxiation, the fur on the back of the animal was removed along with the two hind limbs and tail. An incision was made on the abdomen to remove the intestine, and the spinal cord was cut at the L2 level above the kidneys. The whole pelvic region of the mouse was placed into an organ bath continuously perfused with carbogenated (95% O_2 / 5% CO_2) Krebs buffer (composition, in mM: NaCl 118.4, $NaHCO_3$ 24.9, $CaCl_2$ 1.9, $MgSO_4$ 1.2, KH2PO_4 1.2, glucose 11.7; all acquired from Sigma-Aldrich) which was kept at a temperature of ~35°C to prevent tissue degradation.

Once in the organ bath, the tissue was further dissected under a microscope. The ureters were tied with silk suture (Fisher-Scientific) to stop potential backflow during distension. The pubic symphysis was cut on the left and right sides and removed to expose the underlying urethra. The urethra was cut, and a catheter attached to a syringe pump (New Era Pump Systems, NE-1000) was inserted and tied with suture to prevent leakage. The syringe contained phosphate buffer saline (PBS; Gibco), and the bladder was filled until a certain point where a syringe needle (BD microlance) could be inserted and pierced through the bladder dome without damaging the sensory nerves in the trigone. A double-lumen catheter was then inserted into the hole of the dome and tied with suture. One catheter was attached to a pressure transducer (NL108T2 Digitimer) to monitor intravesical pressure, and another was attached to a tap to allow filling and emptying of the bladder.

Once catheterized, the bladder was distended to 50 mmHg to make sure it was a closed system, as failure to reach this pressure would indicate a leak. The pelvic and hypogastric nerves emerging from the bladder base were dissected into long nerve bundles and inserted into a glass suction electrode (VWR) attached to a Neurolog headstage (NL100AK). The headstage was connected to an AC pre-amp (NL104) to amplify the signal (10 000x), filtered by a pass band filter (NL125) and the 50-60Hz electrical noise was removed by a Humbug (Quest Scientific). The signal was then passed through a 1401 Data Acquisition Interface (Cambridge Electronic Design) and recorded on a computer via Spike2 software (v10.08; Cambridge Electronic Design). Multi-unit afferent activity was quantified using a Spike processor (D130; Digitimer), which counted the number of field potentials passing a threshold set at the beginning of the experiment at twice the baseline noise level. The setup of the preparation is shown in figure 2.1.

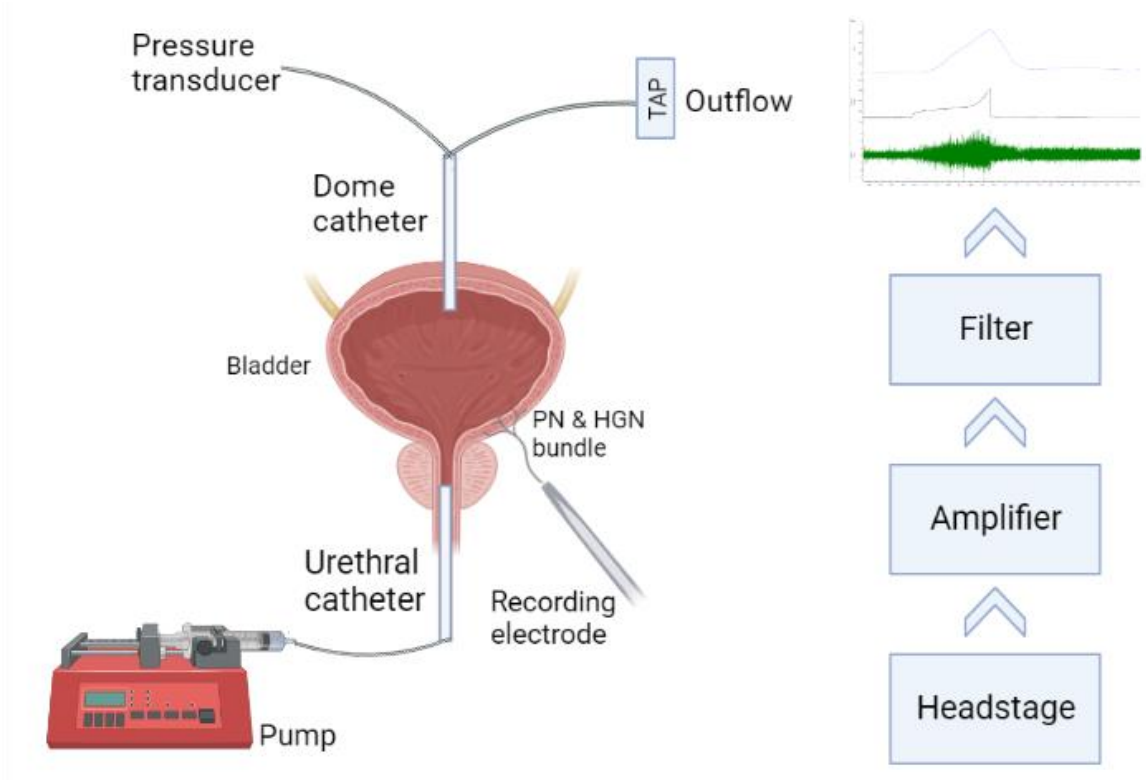


Figure 2.1: A schematic diagram showing the setup of the ex vivo bladder electrophysiology preparation. The bladder was catheterised at the urethra and through the dome. The pelvic nerve (PN) and hypogastric nerve (HGN) bundles were inserted to a glass recording electrode. The technical setup on the right visualises how the input from the pressure transducer and the electrode is modified by the hardware and the Spike2 software to give the example distension image at the top. Figure created using BioRender.

Experimental protocols

In the *ex vivo* bladder electrophysiological recordings, the bladder was stimulated through mechanical (distension) and chemical (application of pharmacological agents) means. The neuronal responses were captured to characterise the effect of the stimulation parameters on afferent nerve activity. The experimental protocols are described in detail below.

Distension

By closing the tap and turning on the syringe pump to fill at a speed of 150µL/min, the bladder slowly distended until a pressure of 50 mmHg was reached. The tap was opened to allow emptying, which caused the pressure to drop immediately. As the bladder filled, the activation of nerve fibres sensitive to mechanical stretch of the bladder wall was captured and visualised as field potentials on Spike2. As the preparation stabilised in the beginning of the experiment, the neuronal responses to distension could vary. Distensions were continued until three reproducible responses were reached concurrently, as shown in figure 2.2 below. Once this was achieved, the experiment was started.

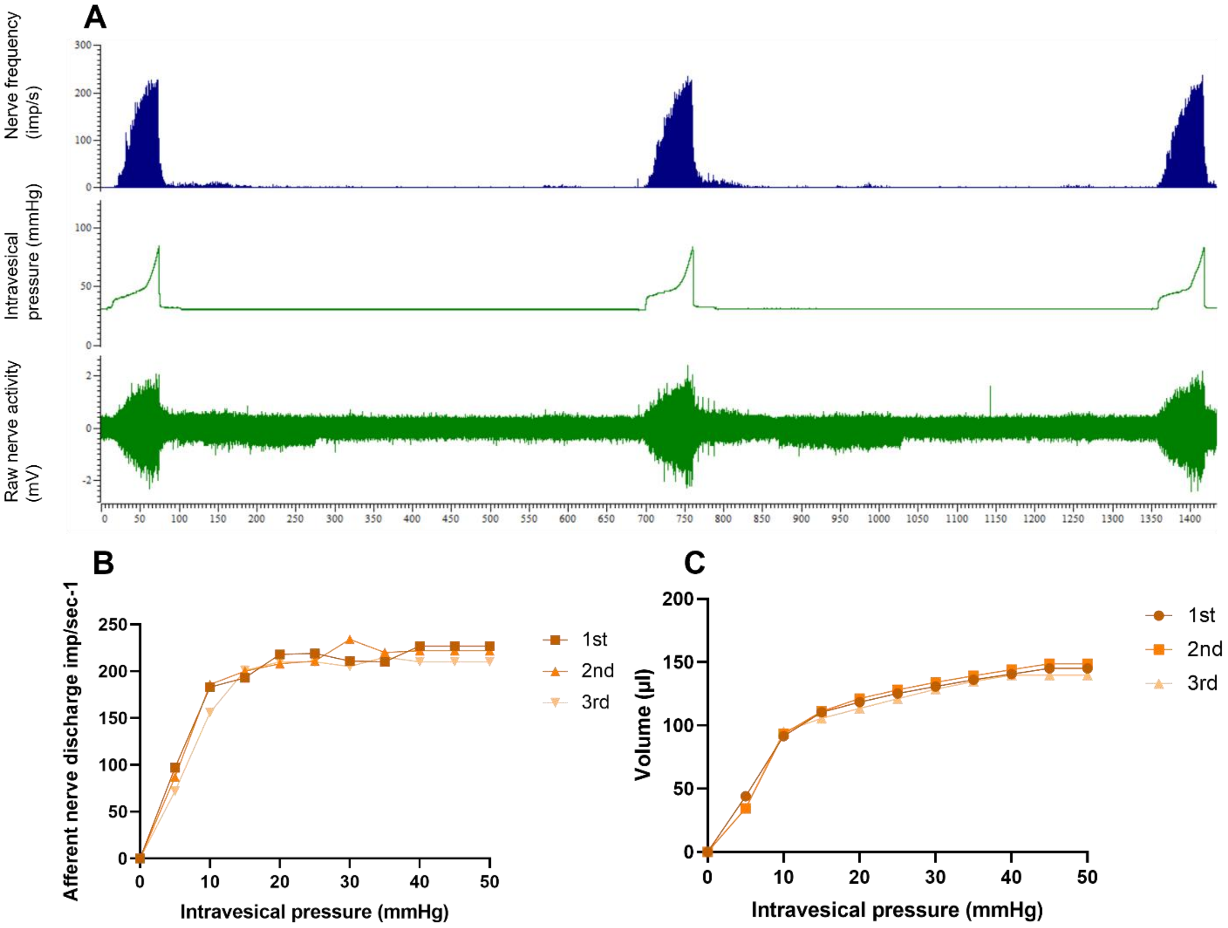


Figure 2.2: Stability and reproducibility of the *ex vivo* bladder-nerve preparation. **A)** an example trace showing three consecutive distensions 10 minutes apart, the preparation is stable over time and the nerve

response is robust and reproducible. The analysis of the three distensions is shown in the graphs. **B)** the afferent nerve response to bladder distension in the first, second and third distension, **C)** the compliance of the bladder or the volume of saline it takes for the intravesical pressure to reach 50 mmHg, which is stable between distensions.

Intraluminal application of BoNTs

The main protocol utilised in this study to characterise the effect of BoNTs on bladder sensation is visualised in figure 2.3 using control data, where the preparation was treated with PBS only. The *ex vivo* bladder electrophysiology assay exhibited reproducibility over 120 minutes, as the response profile of the distension performed 90 minutes into the experiment was like that of the control distension at the beginning (fig 2.4). Bladder compliance appeared to be increased over time in PBS only preparations.

A syringe filled with a BoNT containing solution was connected to the syringe pump and the bladder was distended three times. This was to ensure uptake of BoNT across the urothelium, as a single distension did not seem to produce a robust response. After this, the syringe was replaced with one containing PBS and distensions continued for 90 minutes to assess the effect of BoNT on bladder physiology. Any BoNT within intraluminal fluid was deactivated using Presept (Advanced Sterilization Products) as it came out of the dome catheter.

Due to safety precautions, BoNTs were not applied extraluminally to avoid the production of aerosols.

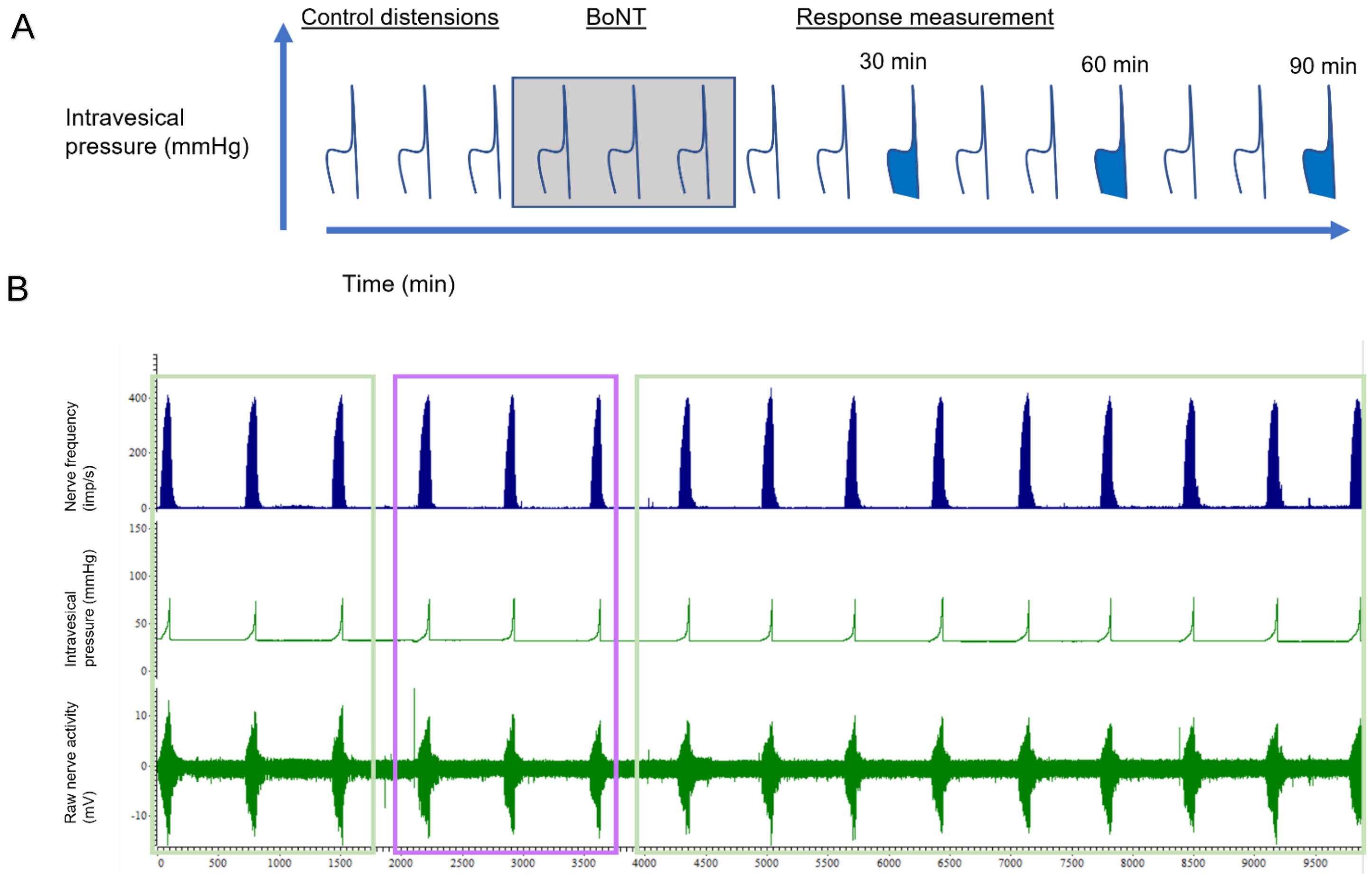


Figure 2.3: Visualisation of the distension protocol used to characterise BoNTs. Above is a cartoon rendition of the experimental design, as well as a representative trace of a control recording. **A)** A schematic diagram of the distension protocol followed. Distensions were defined as rises intravesical pressure to 50 mmHg induced by bladder filling through the syringe pump, which dropped sharply once the tap was opened. All distensions were 10 minutes apart, starting with intravesical phosphate buffered saline (PBS) during the three control distensions, followed by BoNT

diluted in its vehicle PBS (shaded), then back to PBS in the response distensions. The filled blue distensions correspond to the 30, 60 and 90 minute time points post-BoNT used in the analysis. **B)** A representative trace of a control experiment treated with PBS throughout. The green rectangles contain distensions that were conducted with PBS, while the purple rectangles were BoNT distensions.

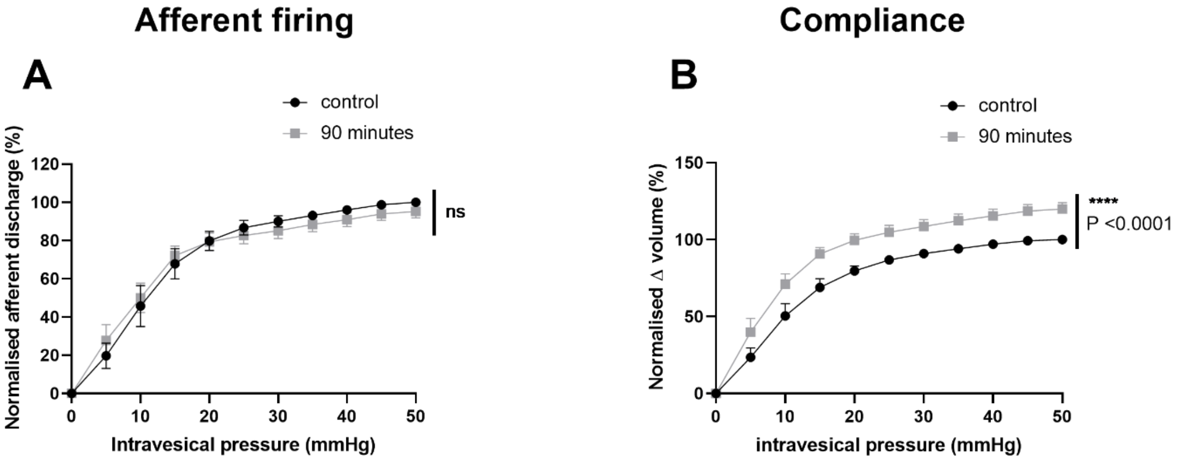


Figure 2.4: Time control preparations that only received PBS throughout the experiment. **A**) there was no significant difference in the mechanosensitivity of afferent nerve fibres in response to distension 90 minutes post-PBS application ($p=0.7445$; $n = 13$; two-way ANOVA). **B**) There appeared to be an increase in bladder compliance post-PBS application ($p<0.0001$; $n = 13$; two-way ANOVA).

Use of pharmacological agents to characterise BoNT mechanisms

In order to better understand the mechanisms of action underlying the effect of BoNTs on bladder sensation, pharmacological modulators were co-applied with BoNTs. Distension experiments were designed to have an internal control prior to BoNT application to show their effect alone, and then alongside BoNT.

To characterise the effect of the purinergic and nitrergic pathways on the BoNT mediated inhibition of bladder mechanosensation, antagonists of P2X receptors and nitric oxide synthase (NOS) were used. L-NAME (Tocris) at the concentration of 1 mM and TNP-ATP (Tocris) at 30 µM were applied using the protocol in figure 2.5. Briefly, PBS distensions were performed at the beginning of the experiment, followed by three distensions with the antagonist dissolved in PBS. After which, three distensions were performed with both BoNT and antagonist in PBS. The experiment was completed with antagonist alone in PBS.

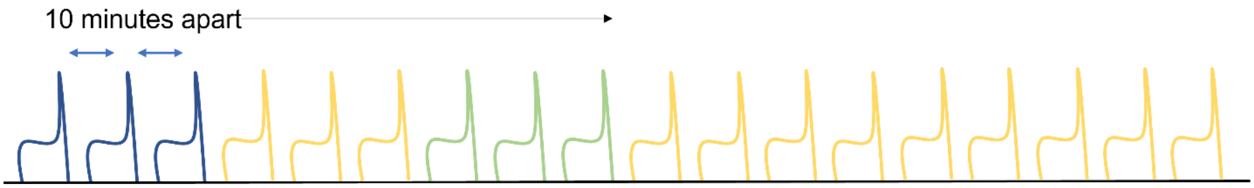


Figure 2.5: Visualisation of the protocol used to characterise pharmacological agents alongside BoNTs. The first three control distensions are shown in blue, the three distensions containing the antagonist alone are in yellow. The green distensions contained the antagonist and BoNT, and the experiment continued with the antagonist alone for 90 minutes.

Extraluminal application of pharmacological agents

To characterise the effect of BoNTs on the chemosensation, responses to capsaicin, a TRPV1 antagonist, was investigated in preparations pretreated with BoNT or PBS (control). 1 µM capsaicin (Sigma-Aldrich) in Krebs solution was perfused into the organ bath using the peristaltic pump. The protocol used in these experiments is shown in below in figure 2.6. After three control distensions and three BoNT distensions, the bladder was filled to a pressure of 20 mmHg and the intraluminal pump was paused to stop further filling and maintain the chosen pressure. After 30 minutes of stabilisation, the input of the perfusion pump was changed to capsaicin-containing Krebs, and the nerve response to extraluminal capsaicin was captured. The input was then changed back to normal Krebs solution.

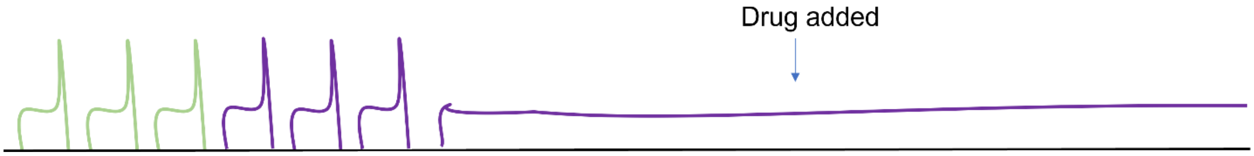


Figure 2.6: a visualisation of the protocol used to characterise the effect of BoNT treatment on the response to extraluminal capsaicin.

2.4 Data analysis

Statistics

All data pertaining to the *ex vivo* bladder electrophysiology assay were captured using Spike2 software (v10.08), including multi-unit nerve firing and intravesical pressure. *N* refers to number of animals, and all data are presented as mean +/- SEM. Following normality testing, parametric statistical tests performed include T-tests, one-way ANOVA and two-way ANOVA using GraphPad Prism v8.0.1. Post-hoc tests were performed as appropriate. Responses from all preparations were kept in the final analysis, assuming the experiment ran with no issues such as equipment failure, tissue failing to fill, the data was included. The specific methods of data extraction and analysis are described in detail below.

Afferent response to distension

Multi-unit afferent firing was quantified by Spike processor (Digitimer D130) which counted the number of spikes over a pre-set threshold (twice the baseline noise level). Neuronal responses to distension were analysed using a custom Spike2 script and represented as number of impulses per second (imp/s). All distension responses were normalised to the third control distension at the beginning of the experiment, which was compared to responses at 30, 60 and 90 minutes following intravesical BoNT treatment. Area under curve was determined from this normalised data.

The effect of BoNTs on afferent firing at low (15 mmHg) and high (50 mmHg) pressures was determined by expressing the responses at these chosen pressures as a percentage of that shown by the control distension at the beginning of the experiments. These pressures were selected due to findings in the literature that designated intravesical pressures <20 mmHg to be considered physiological, and those above that to be painful (Daly et al., 2007).

Bladder compliance

Compliance is a measure of the pressure-volume relationship during bladder filling, or the ability of the bladder wall to accommodate increasing volumes.

$$\text{Volume } (\mu L) = \text{rate } (\mu l/\text{min}^{-1}) \times \text{time}$$

This equation was used to calculate the pressure volume relationship of the bladder using the rate of filling programmed on the intravesical pump, as well as the time taken to reach 50 mmHg. Changes in bladder compliance was also plotted as percentage change as compared to the control distension at the beginning of the experiment. In time control preparations which were treated with PBS alone, across the 90 minutes recorded a shift towards increased compliance was seen (figure 2.4B). This was unexpected as previous comparable studies showed no difference in the pressure-volume relationship despite using longer protocols (>90 minutes) (Collins et al., 2013), which suggests changes seen may be specific to this preparation. This made interpretations of the BoNT treated data more complicated as any changes in compliance may have been BoNT mediated, or due to factors specific to the preparation. A potential way around this may have been to compare vehicle (PBS) treated preparations to the BoNT treated preparations, however, this would not have provided information of the changes within-preparations. Thus, the within-preparation normalisation was continued throughout this thesis with all the BoNT constructs tested, and the results discussed in detail within the Discussion sections.

Immunohistochemistry

Following treatment with Dysport, or PBS for controls, bladders were fixed overnight at room temperature (~20 °C) in 4% paraformaldehyde (PFA), then stored in 1% PFA/PBS solution at 4 °C. The bladder samples were sent to be processed, blocked and sectioned to 8 µm at the University College London pathology lab. Once received, all slides were blinded by taping the identification strip at the top of each slide with opaque tape.

At the start of the experiment, the slides were baked at 45°C overnight, then deparaffinized and hydrated to distilled water. Subsequent steps depended on primary antibody used (SNAP-25 or cleaved SNAP-25), as sections to be stained with the anti-cSNAP-25 antibody required antigen retrieval using EDTA buffer (pH 9) at 98°C for 30 minutes while sections to be stained with anti-SNAP-25 did not. All sections were blocked with 3% hydrogen peroxide (Sigma-Aldrich, 1.07209), followed by 10% horse serum (Sigma-Aldrich, H1270) in Dako buffer.

Sections were incubated at room temperature for one hour with anti-SNAP-25 (rabbit, 1/1000, Sigma-Aldrich, S9684) and overnight with anti-cSNAP-25 (rabbit, 1/1000, provided by Stephane Lezmi from Ipsen Bioinnovation, Les Ulis). All sections (both primary antibodies) were incubated with secondary biotinylated antibody (horse anti-rabbit, 1/400, Vector Labs, BA-1100) for 30 minutes at room temperature. Vectastain Elite ABC HRP Reagent (Vector Labs, PK-7100) was applied for 30 minutes at room temperature to amplify the signal, followed by Haematoxylin for one minute (Sigma-Aldrich, H2126).

At the end of the protocol, slides were then dehydrated with ethanol and the xylene substitute HistoChoice® Clearing Agent (Sigma-Aldrich, H2779), and mounted in mounting media. Negative controls were conducted by following all steps of the protocols except incubation of primary antibody. For positive control, slides containing rat skeletal muscle intoxicated with Dysport were sent by Dr Stephane Lezmi from the Investigative Pathology Lab at Ipsen Bioinnovation, Les Ulis, France. The results of the negative and positive control staining are shown in figure 2.7.

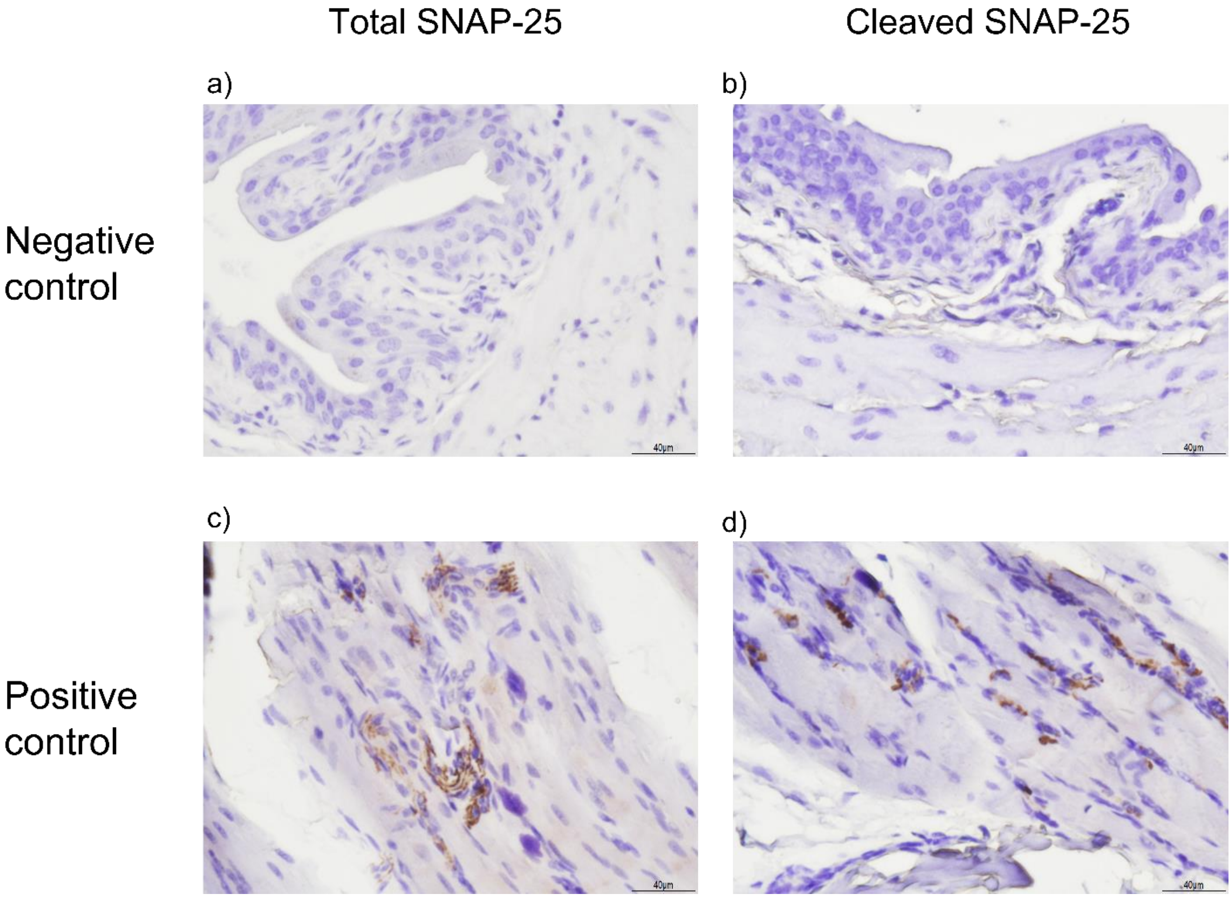


Figure 2.7: A representative figure showing images of negative and positive controls following immunohistochemistry, showing haematoxylin-stained nuclei and brown staining indicating DAB immunoreactivity of SNAP-25 and cSNAP-25 in rat neuromuscular junctions in positive controls.

CHAPTER 3 – THE EFFECT OF THE BONT/A COMPLEX AND ITS CONSTITUENT SUBDOMAINS ON BLADDER MECHANOSENSITIVITY

INTRODUCTION

Urgency is a symptom of overactive bladder (OAB) and is defined as the strong, unavoidable urge to urinate that cannot be deferred and may be followed by incontinence (Abrams et al., 2003). OAB has severe consequences on patients' quality of life, by limiting their daily activities and affecting their mental health to the extent that OAB is associated with increased rates of depression and anxiety (Nicolson et al., 2008; Sexton et al., 2011). BoNT/A has been used successfully in the clinic for decades in the treatment of urological disorders including OAB, the clinical trials for which have shown patient reports of reductions in urgency (Chapple et al., 2013; Dmochowski et al., 2010). These findings were unexpected, as the rationale for BoNT/A use in the bladder was predicated on its ability to paralyse neuromuscular junctions. The mechanism of action underlying the alterations in sensory signalling remain unclear. Investigators started to untangle these research questions by looking for the presence of the protein receptor SV2 and SNARE target SNAP-25 in the bladder, however, this has led to mixed results in the literature which are discussed in depth in chapter one. This leaves a significant gap in our understanding of the mechanisms responsible for the sensory modulation of BoNT/A in the bladder.

There are other aspects of BoNT/A entry that have yet to be explored in the bladder. Clostridium botulinum produces BoNTs as part of multi-protein complexes, which include haemagglutinins and non-toxic non-haemagglutinin accessory proteins (NTNH). Due to their lack of participation in SNARE cleavage, there has not been much discussion on their role in the BoNT/A dependent modulation of sensory signals in the bladder field. However, there may be a potential role for NTNH and haemagglutinin proteins to aid in the entry of BoNT/A into the bladder wall. Previous studies have found that the complexing accessory proteins facilitate the function of BoNT/A by disrupting the intestinal barrier, as Matsumara et al (2008) found haemagglutinin proteins to increase transcellular permeability by opening tight junctions (Matsumura et al., 2008). This facilitated increased transcellular transport of the BoNT/A molecule and potentially increased the lethality of the molecule (Ghosal et al., 2018; Matsumura et al., 2008). Interestingly, other studies have found no difference between the oral toxicity of the pure BoNT/A molecule versus the BoNT/A complex including the NTNH and haemagglutinins (Maksymowych et al., 1999). In fact, Couesnon et al (2007) found BoNT/A to cross intestinal layers in an accessory protein independent manner, showing that transcytosis depended on the heavy chain (HC) domain of BoNT/A binding to gangliosides expressed on intestinal cells (Couesnon et al., 2007). This is supported by Maksymowych & Simpson (2004) who termed the HC domain as a "carrier domain" due to its ability to cross epithelial layers (Maksymowych & Simpson, 2004). These findings suggest that BoNT/A dependent entry through epithelial layers may depend on the NTNH and haemagglutinin proteins, or solely on the HC domain. In the context of bladder sensation, increased transcytosis across the urothelium and internalisation into the suburothelial layer may allow BoNT/A to disrupt the urothelial-associated sensory web and subsequently modulate bladder sensation. The potential roles of the NTNH and haemagglutinin accessory proteins, as well as the HC domain, on afferent signalling is studied in detail within this chapter using recombinantly produced constructs of BoNT/A.

Advances in the methods of recombinant protein design and production has accelerated innovation in the field of BoNT research. These methods use *Escherichia coli* to produce BoNTs instead of *Clostridium botulinum*, bypassing the safety risks of spore production and the extensive and laborious purification processes (Dolly et al., 2011). Previous studies have produced the subdomains of BoNT/A separately, creating functional constructs of the catalytic light chain (LC) (Zhou et al., 1995), translocation domain (HN) (Lacy & Stevens, 1997) and receptor binding domain (HC) (Clayton et al., 1995). These methods were further developed to allow the production of a construct of BoNT/A without the HC domain, termed LHn/A (Chaddock et al., 2000a; Chaddock et al., 2000b).

This chapter presents, for the first time, a systematic study of the mechanisms of entry employed by the BoNT/A complex and its constituent subdomains, and how this may affect the BoNT/A dependent inhibition of bladder sensory signalling reported previously (Collins et al., 2013). The light and heavy chain subdomains of BoNT/A were investigated separately to assess their relative importance to the modulation of bladder sensation. The effect of the entire BoNT/A complex (>500 kDa) as produced by *Clostridium botulinum* was compared to the pure neurotoxin alone (150 kDa), to better understand the role of the NTNH and haemagglutinin proteins.

As the potent neuroparalytic activity of BoNT/A is dependent on its HC domain targeting cholinergic neurons, retargeting of the catalytic light chain to cells of interest may be achieved by changing the receptor binding region. Increased release of neurotrophic factors such as nerve growth factor (NGF) in urological diseases such as IC/PBS has been shown to result in increased expression of its receptor, tropomyosin receptor kinase A (TrkA) (Qiao & Vizzard, 2002). A construct of BoNT/A targeting the TrkA receptor expressed on sensory neurons was investigated to understand whether directly targeting sensory neurons may cause a more pronounced inhibitory effect than that shown by the original BoNT/A molecule, which may provide a more targeted treatment of IC/PBS.

MATERIALS AND METHODS

Ex vivo extracellular electrophysiology recordings from the mouse bladder

The *ex vivo* extracellular electrophysiology method is described in detail in chapter 2. Briefly, the urethral catheter was connected to a syringe pump that was programmed to fill the bladder at a rate of 150µL/min. Once 50 mmHg was reached, the tap connected to the dome catheter was opened to allow bladder emptying. A nerve bundle containing pelvic and hypogastric nerves was inserted into a glass electrode to facilitate capture of afferent nerve responses to bladder stimulation. All ramp distensions were performed 10 minutes apart, and each preparation was allowed to stabilise for at least 30 minutes before recording started.

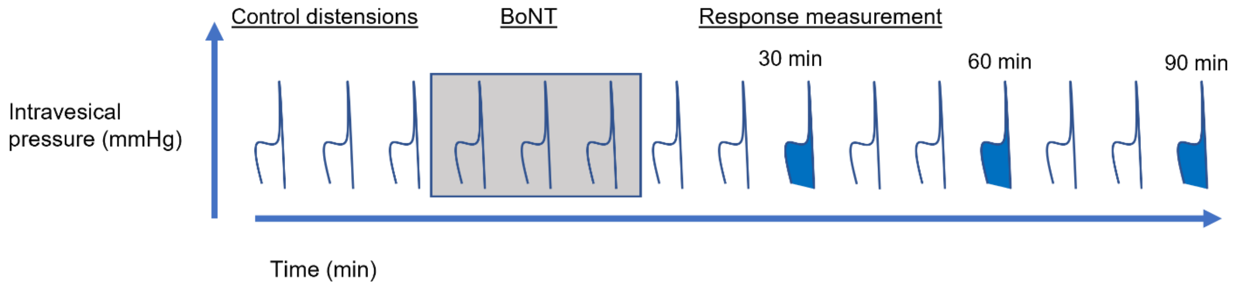


Figure 3.1: A schematic diagram of the distension protocol followed. Distensions were defined as rises in intravesical pressure to 50 mmHg induced by bladder filling through the syringe pump, which dropped sharply once the tap was opened. All distensions were 10 minutes apart, starting with intravesical phosphate buffered saline (PBS) during the three control distensions, followed by BoNT in vehicle PBS (shaded), then back to PBS in the response distensions. The blue filled distensions correspond to the 30, 60 and 90 minute time points post-BoNT used in the analysis.

Distensions were performed until the distension-induced nerve responses were reproducible, the protocol followed is shown above in figure 3.1. Following three reproducible 50 mmHg distensions using PBS, full-length BoNT/A or its associated fragments were applied intravesically using a syringe pump, the bladder was distended three times at a speed of 150 µL/min to 50 mmHg. After which, distensions were continued with PBS for nine distensions 10 minutes apart.

Preparation of BoNT/A and domain fragments

Dysport® was supplied by Ipsen Bioinnovation. The native 150 kDa BoNT/A was purchased from List Biologicals, herein designated ‘native BoNT/A’ or nBoNT/A as it is produced by *Clostridium botulinum* and purified from the larger protein complex. Recombinant BoNT/A (or rBoNT/A) and its constituent subdomains including HC/A, LHn/A and LC/A were produced in-house at Ipsen, the gene sequences were cloned into vectors, expressed in *E. coli* then purified and activated. The structures of these proteins are shown below in figure 3.2. BoNTs were stored at -80°C, on each day before use vials were defrosted and diluted to the target concentration in the phosphate buffered saline (PBS) vehicle in a Class 2 Microbiological Safety Cabinet following necessary safety protocols.

Preliminary experiments revealed the Dysport concentration to provide robust, reproducible responses to be 100U/ml. The total toxin amount was calculated to be 3.6pM (Field et al., 2018) so to facilitate accurate comparison, all other BoNTs were applied at 3.6 pM, as well as higher and lower concentrations.

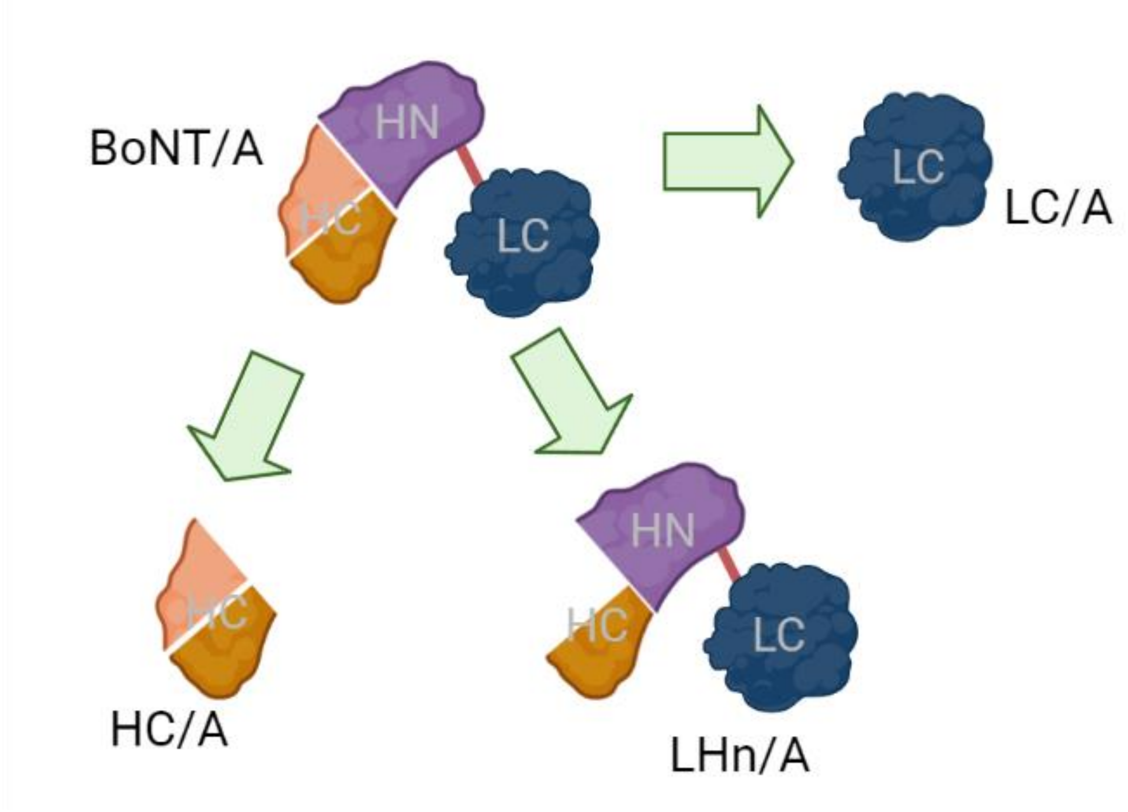


Figure 3.2: a schematic diagram representing the BoNT/A and its subdomains that were investigated in this study. The heavy chain (HC/A; pink and orange representing the HCc and HCn domains respectively) and light chain (LC/A; blue) were tested as well as LHn/A, which is missing the SV2 binding region (HCc domain; pink). Figure created using BioRender.

Data analysis

Neuronal responses to ramp distension were extracted from Spike2 files using a custom script. Responses at 30, 60 and 90 minutes were normalised to the third reproducible control distension at the start of the experiment. Area under the curve was determined from this normalised data. Peak firing at 15 mmHg and 50 mmHg at 30, 60 and 90 minutes were compared to that of the control distension at the start of the experiment. *N* refers to number of animals, and all data are presented as mean +/- SEM. Statistical tests performed include T-tests, one-way ANOVA and two-way ANOVA using GraphPad Prism v8.0.1.

Immunohistochemistry (IHC)

Bladders pretreated with Dysport and PBS (control) were stained for presence of total and cleaved SNAP-25, to investigate whether SNAP-25 cleavage has a role in modulating the afferent responses to distension. The IHC protocol as well as the validation of the anti-SNAP-25 (Sigma-Aldrich) and anti-cleaved SNAP-25 (produced in-house) antibodies are described in detail in chapter 2.

RESULTS

3.1 Effect of the BoNT/A complex (Dysport) on bladder mechanosensitivity

Intravesical application of 100U/ml Dysport led to a significant reduction in distension induced afferent firing over the 90-minute protocol (figures 3.3 and 3.4A; *n* = 9; p<0.0001). Bladder compliance appeared to be increased following Dysport treatment (figure 3.4B; *n* = 9; p=0.0003). To investigate whether there was a temporal element in the Dysport induced inhibition, the level of inhibition at the measured time points were compared. 30 minutes after treatment, peak nerve firing at 50 mmHg was reduced by 18.2% (+/- 6.23%), while by 90 minutes post-treatment nerve firing was reduced by 32.5 % (+/- 10.7%). However, the data shows no difference between the action of Dysport on units firing at the physiological pressure (15 mmHg) and at the nociceptive pressure (50 mmHg) (figure 3.5, p = 0.1003).

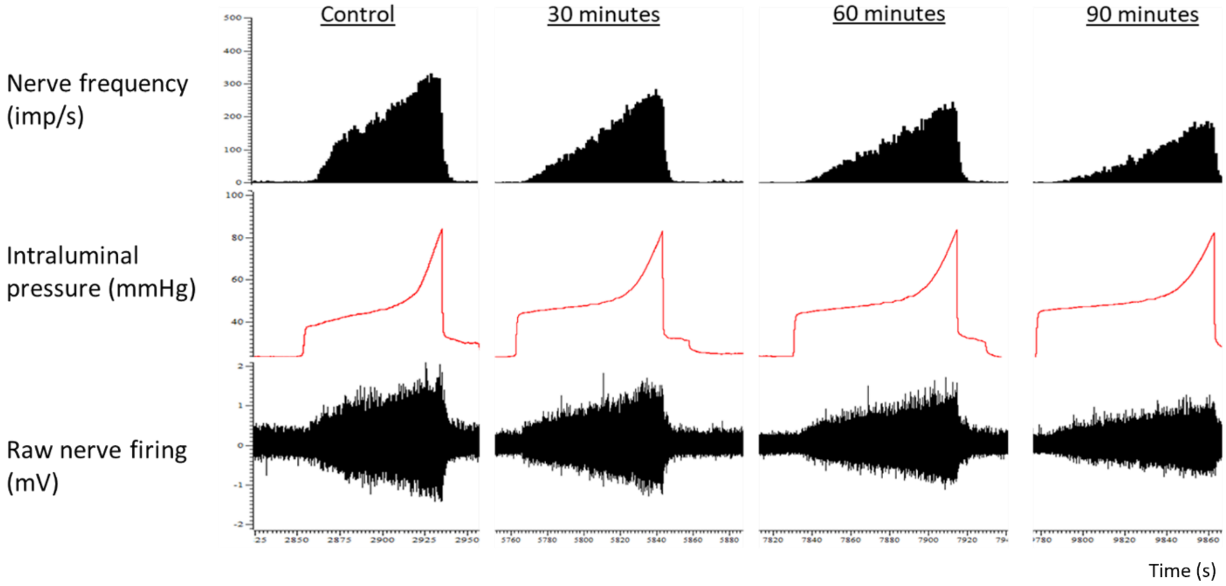


Figure 3.3: an example trace showing the response of afferent nerves to distension prior to (control) and 30, 60 and 90 minutes after application of 100 U/ml Dysport.

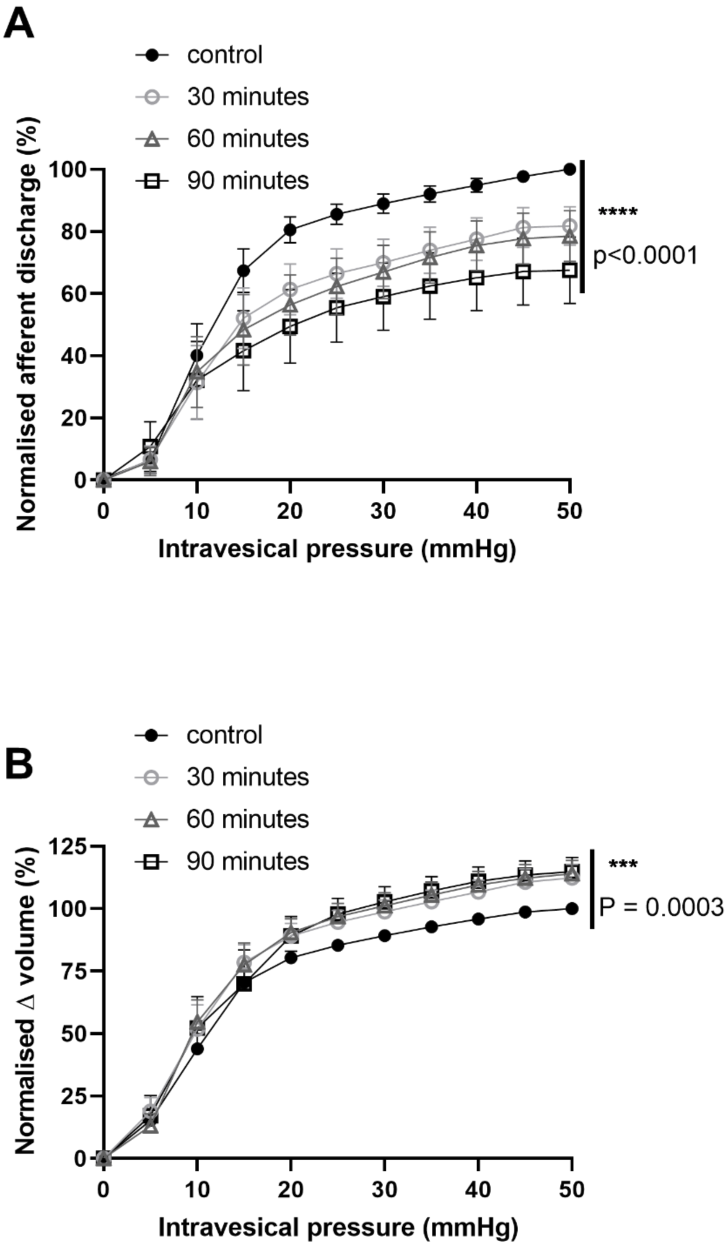


Figure 3.4: Intravesical Dysport treatment led to significant decreases in bladder mechanosensitivity and increases in the pressure-volume relationship. **A)** Afferent responses to distension were significantly reduced 30, 60 and 90 minutes after treatment ($p<0.0001$, $n = 9$, two-way ANOVA). **B)** The pressure-volume

relationship was significantly increased after intravesical Dysport compared to control (p = 0.0003; n = 9; two-way ANOVA).

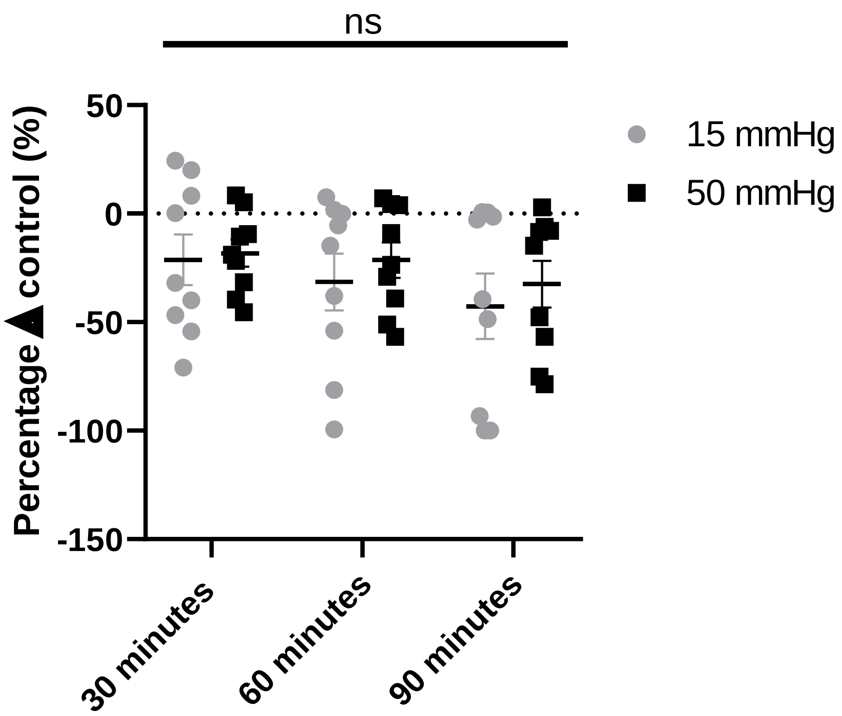


Figure 3.5: Percentage change of peak firing at 15 mmHg and 50 mmHg showed no significant difference between the early and later time points (p = 0.1003; *n* = 9; two-way ANOVA).

3.2 Effect of 150 kDa nBoNT/A on bladder mechanosensitivity

To investigate the contribution of the non-toxic non-haemagglutinin (NTNH) proteins that complete the BoNT/A complex on bladder mechanosensitivity, the 150 kDa BoNT/A molecule was applied alone. This native 150 kDa BoNT/A (or nBoNT/A) was purified from *Clostridium botulinum* type A and has been shown to cleave SNAP-25 at a similar potency as the full complex (Hunt et al., 2010).

Intravesical application of 3.6pM nBoNT/A led to a decrease in the mechanical responsiveness of the nerves, as over the 90-minute protocol, distension induced afferent firing was significantly reduced (figures 3.6 and 3.7A; $p<0.0001$; n = 5; two-way ANOVA). Bladder compliance was significantly increased following treatment with nBoNT/A (figure 3.7B; $p < 0.0001$; n = 5; two-way ANOVA). 30 minutes after treatment, peak nerve firing at 50 mmHg was reduced by 17.75% (+/- 9.16%), while by 90 minutes post-treatment nerve firing was reduced by 29.8 % (+/- 10.24%). Interestingly, when the level of inhibition between the time points were compared, there was no significant difference between the percentage decrease at 30, 60 or 90 minutes, at the low threshold pressure and at the high threshold pressure (figure 3.8, $p = 0.1401$).

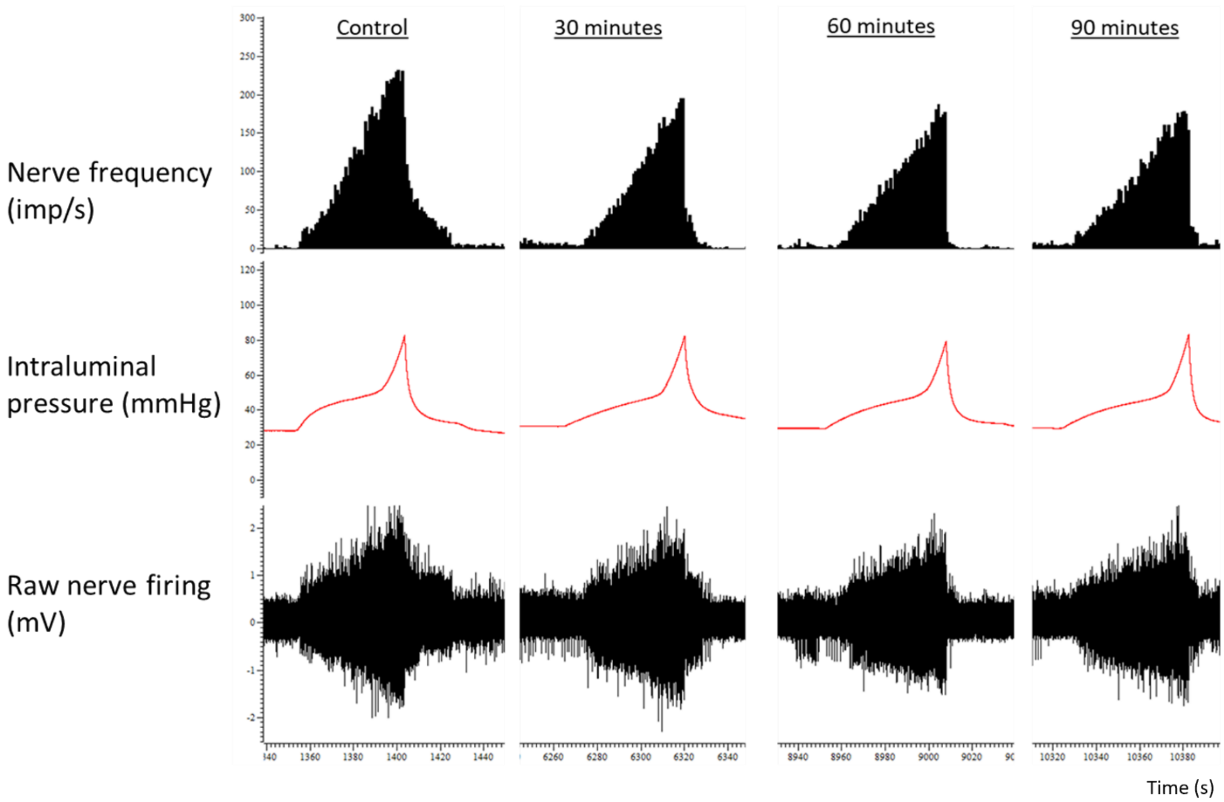


Figure 3.6: an example trace showing the response of afferent nerves to distension prior to (control) and 30, 60 and 90 minutes after application of nBoNT/A.

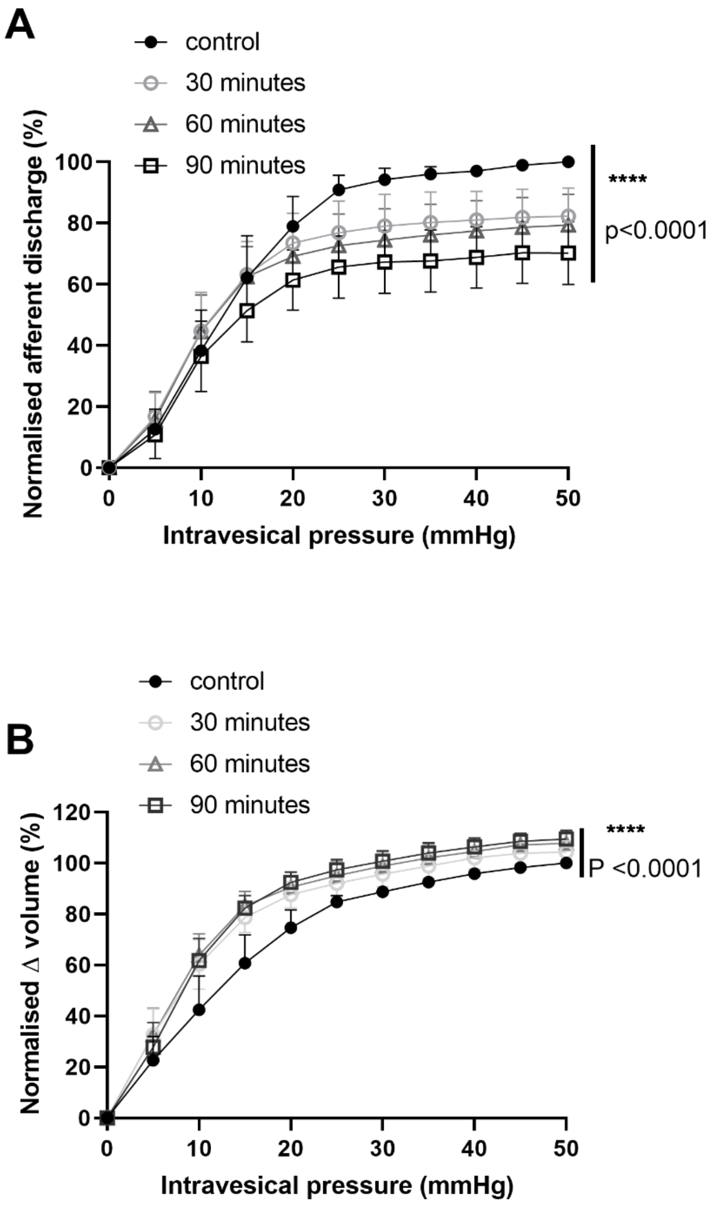


Figure 3.7: Intravesical nBoNT/A treatment led to significant decreases in bladder mechanosensitivity**. A)** Afferent responses to distension were significantly reduced 30, 60 and 90 minutes after treatment

(p<0.0001, *n* = 5; two-way ANOVA). **B)** the pressure-volume relationship was significantly increased after intravesical nBoNT/A compared to control (p = 0.0001; *n* = 5; two-way ANOVA).

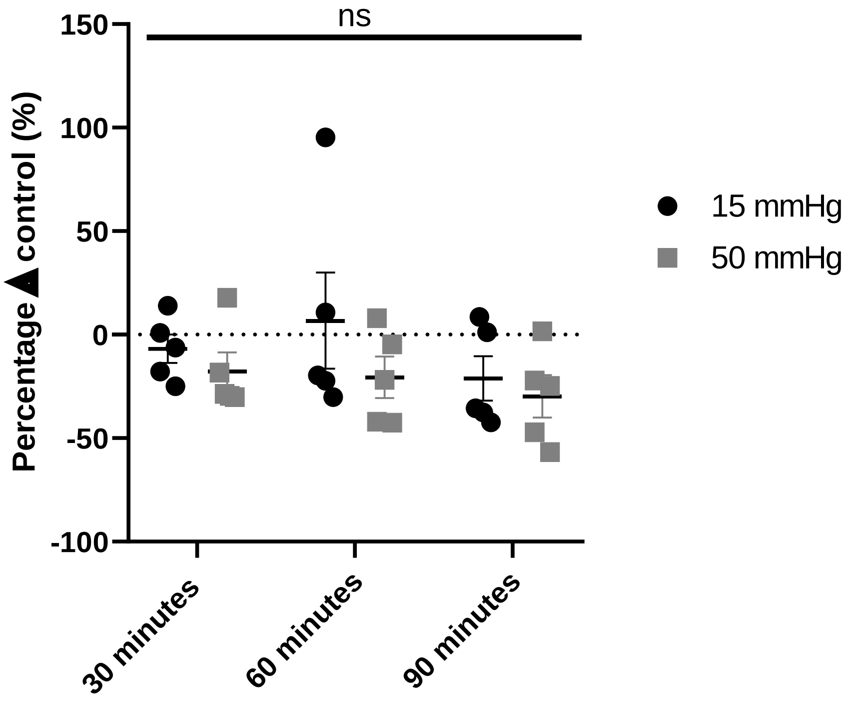


Figure 3.8: Percentage change of peak firing at 15 mmHg and 50 mmHg showed no significant difference between the early and later time points (p = 0.3436; *n* = 5; two-way ANOVA).

3.3 Effect of 300pM nBoNT/A on bladder mechanosensitivity

To further characterise the effects of nBoNT/A on distension induced afferent firing, a higher concentration was intravesically instilled. 300 pM nBoNT/A significantly decreased afferent firing as by 90 minutes following treatment, only 67.6% (+/- 7.562%) of the nerve response remained (figure 3.9A; $p < 0.0001$; $n = 4$). Bladder compliance also showed a significant increase (figure 3.9B; $p = 0.02$; $n = 4$). To look separately at the effect of 300 pM nBoNT/A on low threshold and high threshold firing, the peak responses at each respective pressure was compared to that of the control distension at the beginning of the experiment. Peak firing at both 15 and 50 mmHg did not change over time ($p = 0.2324$; figure 3.10).

Increasing the concentration of nBoNT/A did not affect its inhibition of afferent firing, as there was no significant difference between the response to 3.6pM and 300 pM (figure 3.11A; $p = 0.0704$). Compared to control, distension induced firing was significantly reduced by 3.6pM (figure 3.11A; $p = 0.0292$) and 300 pM ($p = 0.0034$). The percentage decrease at 15 mmHg and 50 mmHg was also not different between the tested concentrations (figure 3.11B; $p = 0.9964$).

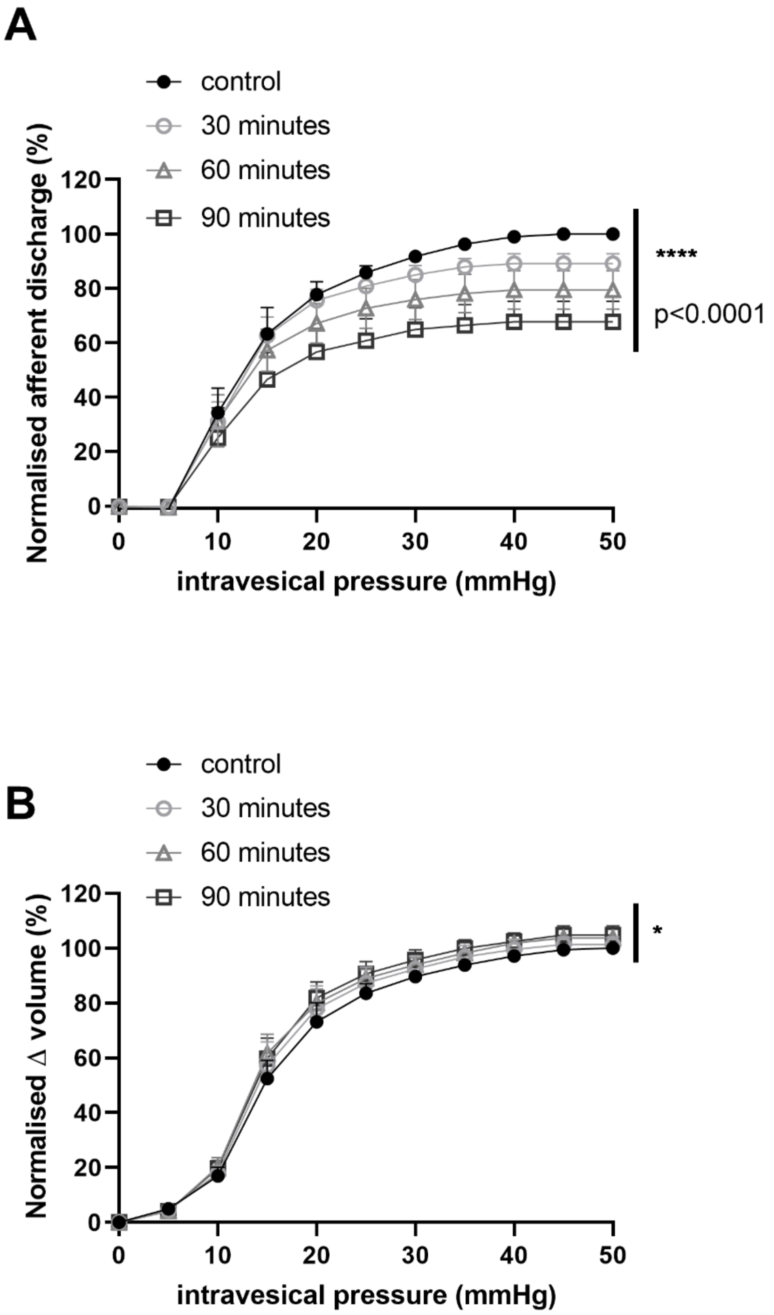


Figure 3.9: The effect of 300 pM nBoNT/A on bladder mechanosensitivity. **A)** Afferent responses to distension were significantly reduced 30, 60 and 90 minutes after treatment ($p<0.0001$, n = 4; two-way ANOVA). **B)** the pressure-volume relationship was significantly increased after intravesical nBoNT/A compared to control ($p = 0.0220$; n = 4; two-way ANOVA).

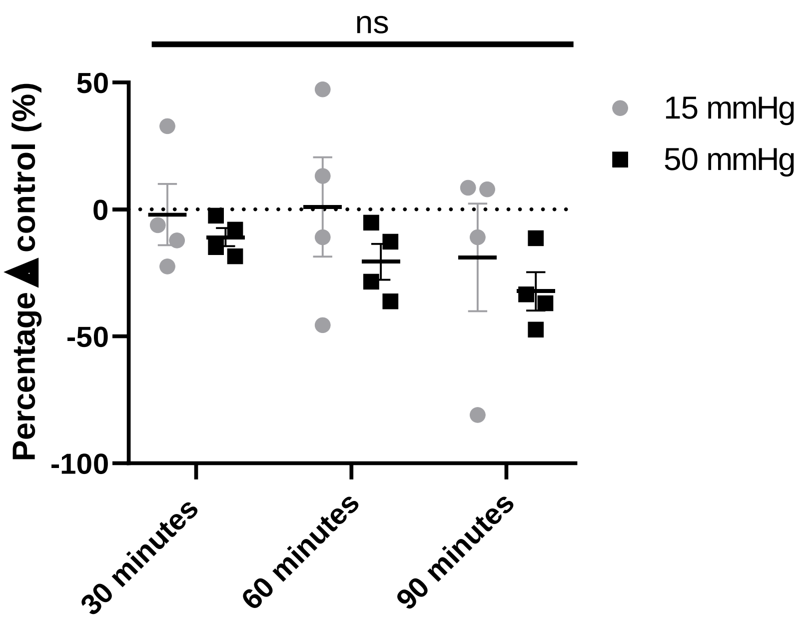


Figure 3.10: Peak firing at 15 mmHg and 50 mmHg showed no significant difference between the early and later time points (p = 0.2324; *n* = 4; two-way ANOVA).

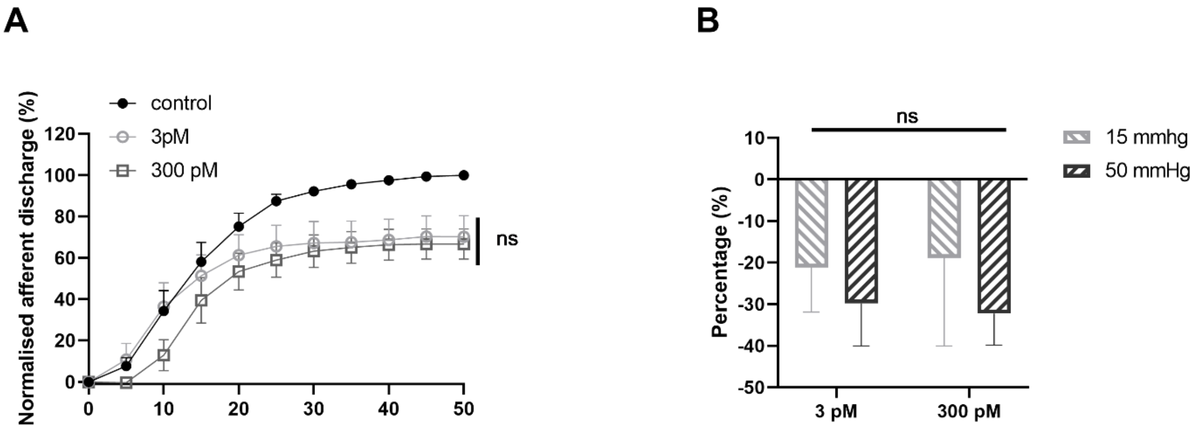


Figure 3.11: The effect of increasing concentrations of nBoNT/A on bladder mechanosensation. **A)** There appeared to be no difference between nerve responses following treatment with increasing concentrations of nBoNT/A (p = 0.0704; two-way ANOVA). **B)** There was no difference in the effect of 3 pM and 300 pM nBoNT/A on low threshold (15 mmHg) and high threshold (50 mmHg) firing (p = 0.9964; two-way ANOVA).

3.4 Effect of recombinant BoNT/A (rBoNT/A) on bladder mechanosensitivity

The mechanism of BoNT/A entry into the bladder wall was investigated in this thesis by taking apart the BoNT/A molecule into its constituent domains, to show which domains are necessary to induce the BoNT/A dependent inhibition of bladder mechanosensitivity. The data shown below describes the effects of the various BoNT/A constructs on distension-induced afferent firing, to define the activity of the heavy chain (HC/A) and the light chain (LC/A). As these molecules were recombinant and expressed in *E. coli*, compared to the native BoNT/A molecules produced by *C. botulinum*, there was a possibility that manufacturing methods could constitute a confounding variable that could make data interpretation difficult. Therefore, the full length recombinant BoNT/A was tested in this model to facilitate comparison with the recombinant subdomains.

In figure 3.12 below, a representative recording of distension induced responses following treatment with rBoNT/A shows afferent nerve firing decreased over time. The data analysis showed this was significant ($p<0.0001$; figure 3.13A; $n = 6$). 30 minutes after treatment, peak nerve firing at 50 mmHg was reduced by 26.97% (+/- 10.24%) while by 90 minutes post-treatment nerve firing was reduced by 28.52% (+/- 9.85%). There did not appear to be a time-dependent inhibition of nerve firing following treatment with rBoNT/A at both 15 and 50 mmHg ($p = 0.5200$; figure 3.14). Bladder compliance appeared to be increased following rBoNT/A treatment (figure 3.13B; $p < 0.0001$; $n = 6$).

There appeared to be no significant difference in the inhibition of nerve firing induced by rBoNT/A when compared to the previously tested formulations of BoNT/A produced by *C. botulinum* Dysport and nBoNT/A (figure 3.15A; $p = 0.4507$). The effect of BoNT/A on physiological and nociceptive signalling remained the same despite the differences in manufacturing (figure 3.15B)

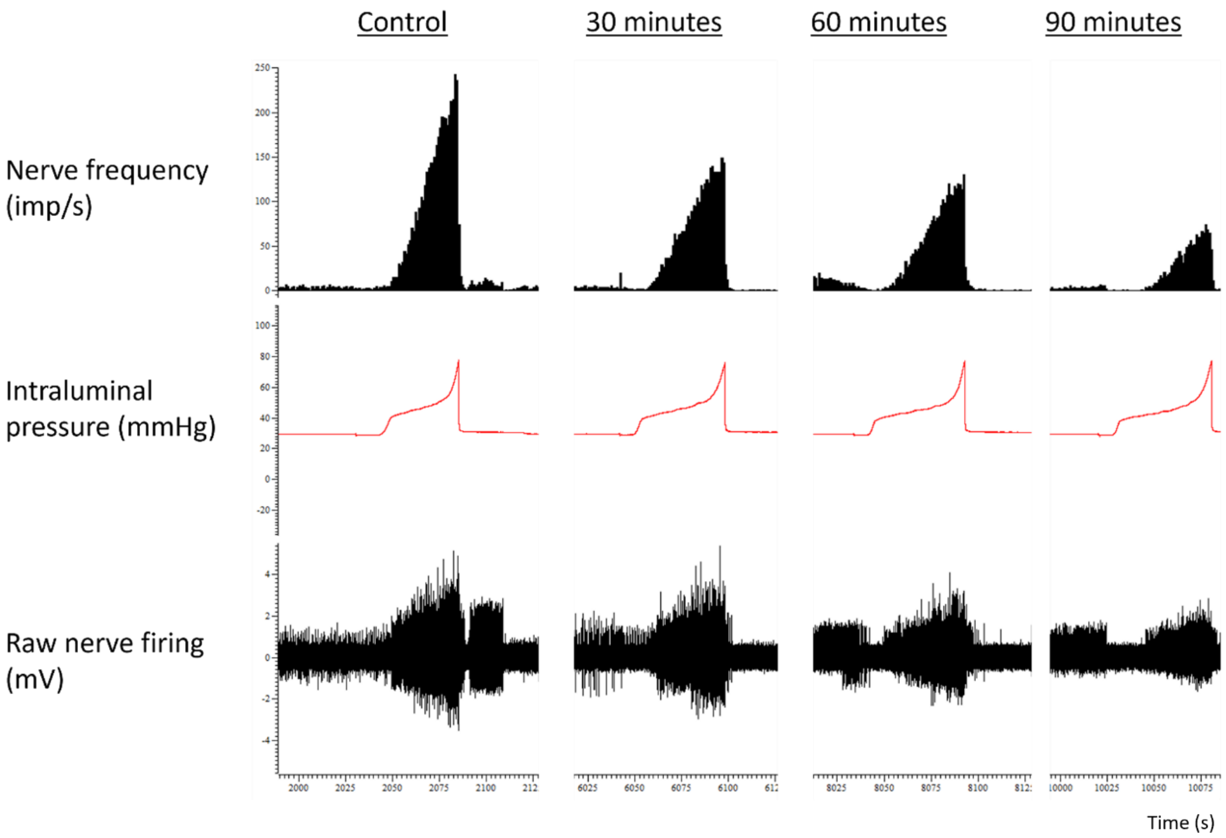


Figure 3.12: an example trace showing the response of afferent nerves to distension prior to (control) and 30, 60 and 90 minutes after application of rBoNT/A.

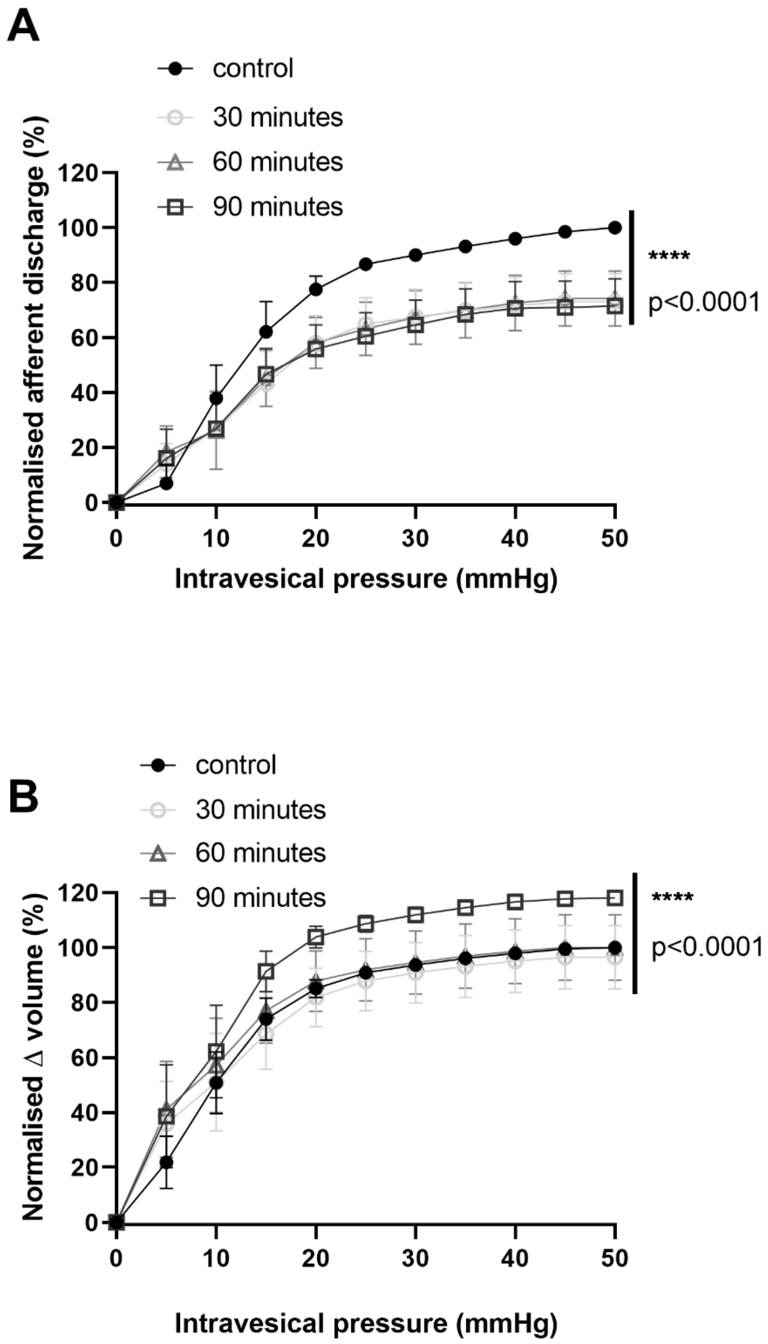


Figure 3.13: rBoNT/A treatment significantly reduced distension induced afferent firing. **A)** Afferent responses to distension were significantly reduced 30, 60 and 90 minutes after treatment ($p<0.0001$, *n* = 6; two-way ANOVA). **B)** the pressure-volume relationship was significantly increased after intravesical nBoNT/A compared to control ($p<0.0001$; *n* = 6; two-way ANOVA).

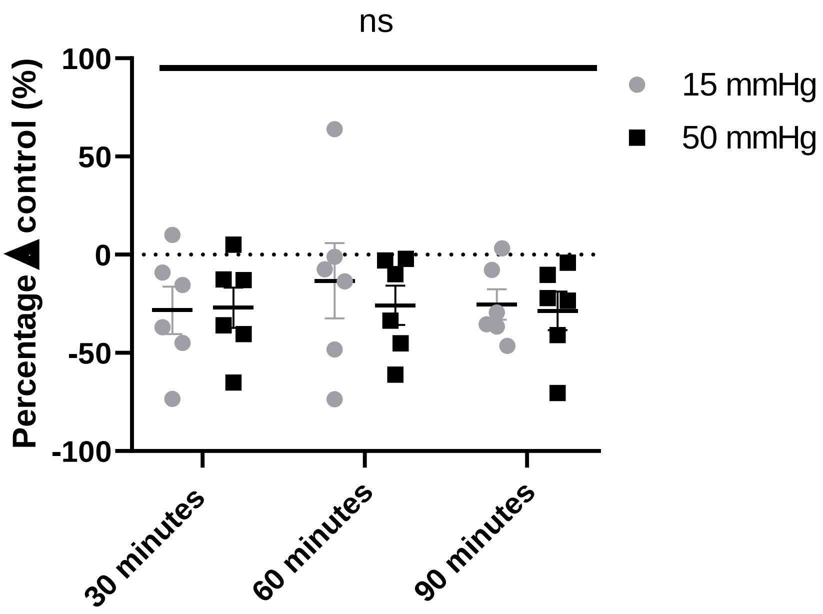


Figure 3.14: Percentage change of peak firing at 15 mmHg and 50 mmHg showed no significant difference between the early and later time points (p = 0.5200; *n* = 6; two-way ANOVA).

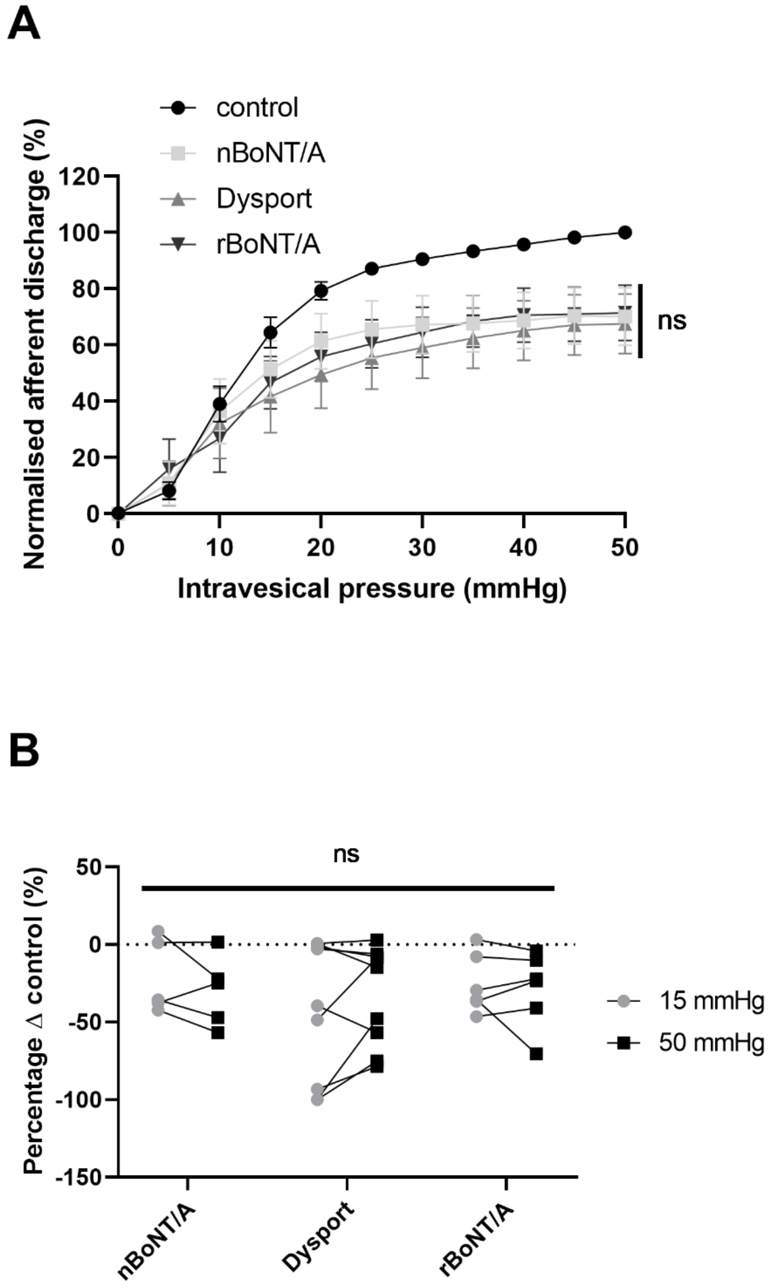


Figure 3.15: There was no difference in the activity of nBoNT/A, Dysport or rBoNT/A on distension induced firing. **A)** all three formulations of BoNT/A significantly inhibited bladder mechanosensitivity (p <0.0001; nBoNT/A *n* = 5; Dysport *n* = 9; rBoNT/A *n* = 6; two-way ANOVA), however there was no difference between them (p 0.4507; two-way ANOVA). **B)** the BoNT/A formulations tested had similar effects on physiological

and nociceptive signalling as there was no significant difference between the early and later time points ($p = 0.7009$; nBoNT/A *n* = 5; Dysport *n* = 9; rBoNT/A *n* = 6; two-way ANOVA).

3.5 Effect of LHn/A on bladder mechanosensitivity

Next, the contribution of the receptor binding region, or the H_C domain, on the BoNT/A mediated inhibition of bladder mechanosensitivity was investigated. This was achieved by applying LHn/A, a recombinant protein with the H_C domain removed and assessing its effect on distension induced firing. As shown in figure 3.16 below, LHn/A inhibited nerve responses to distension in a time dependent manner.

Despite the lack of receptor binding region, LHn/A was able to enter the bladder wall and inhibit bladder mechanosensitivity (figure 3.17A; $p = 0.0003$; $n = 7$; two-way ANOVA). Interestingly, bladder compliance appeared to be increased following LHn/A treatment (figure 3.17B; $p < 0.0005$; $n = 7$). 30 minutes after treatment, peak nerve firing at 50 mmHg was reduced by 6.06% (+/- 8.9%), while by 90 minutes post-treatment nerve firing was reduced by 14.83 % (+/- 21.41%). When the level of inhibition between the time points were compared, there was no significant difference between the percentage decrease at the physiological (15 mmHg) and nociceptive (50 mmHg) pressures ($p = 0.7375$; figure 3.18). These findings suggest nerve populations responsive to these pressures were equally inhibited by LHn/A.

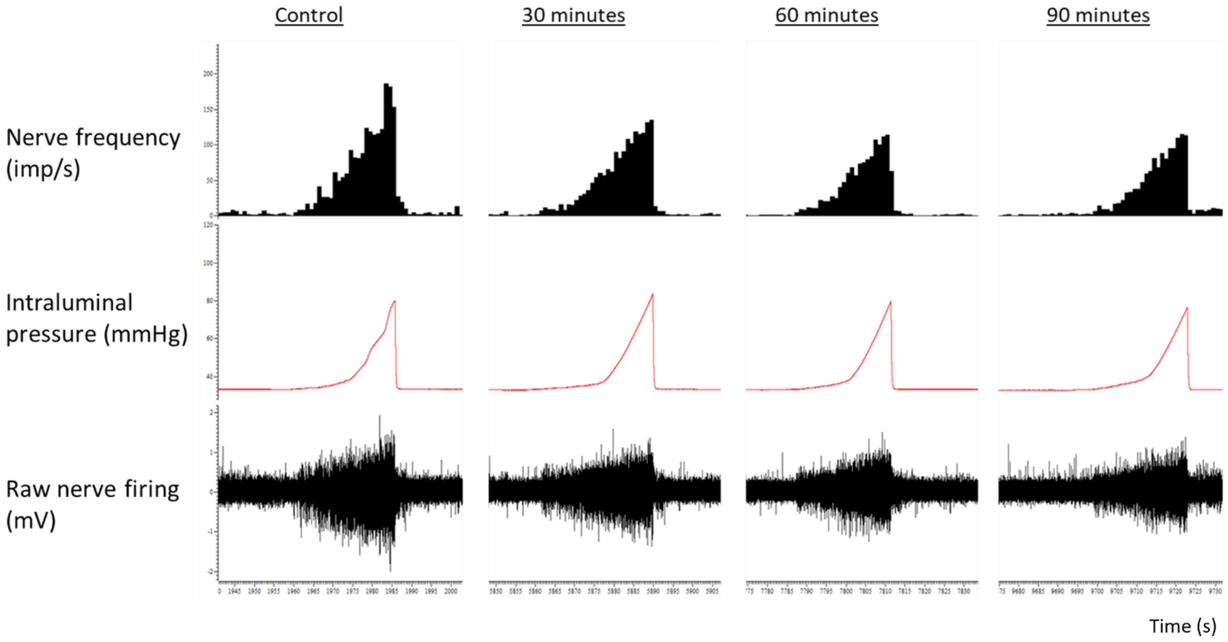


Figure 3.16: an example trace showing the response of afferent nerves to distension prior to (control) and 30, 60 and 90 minutes after application of LHn/A.

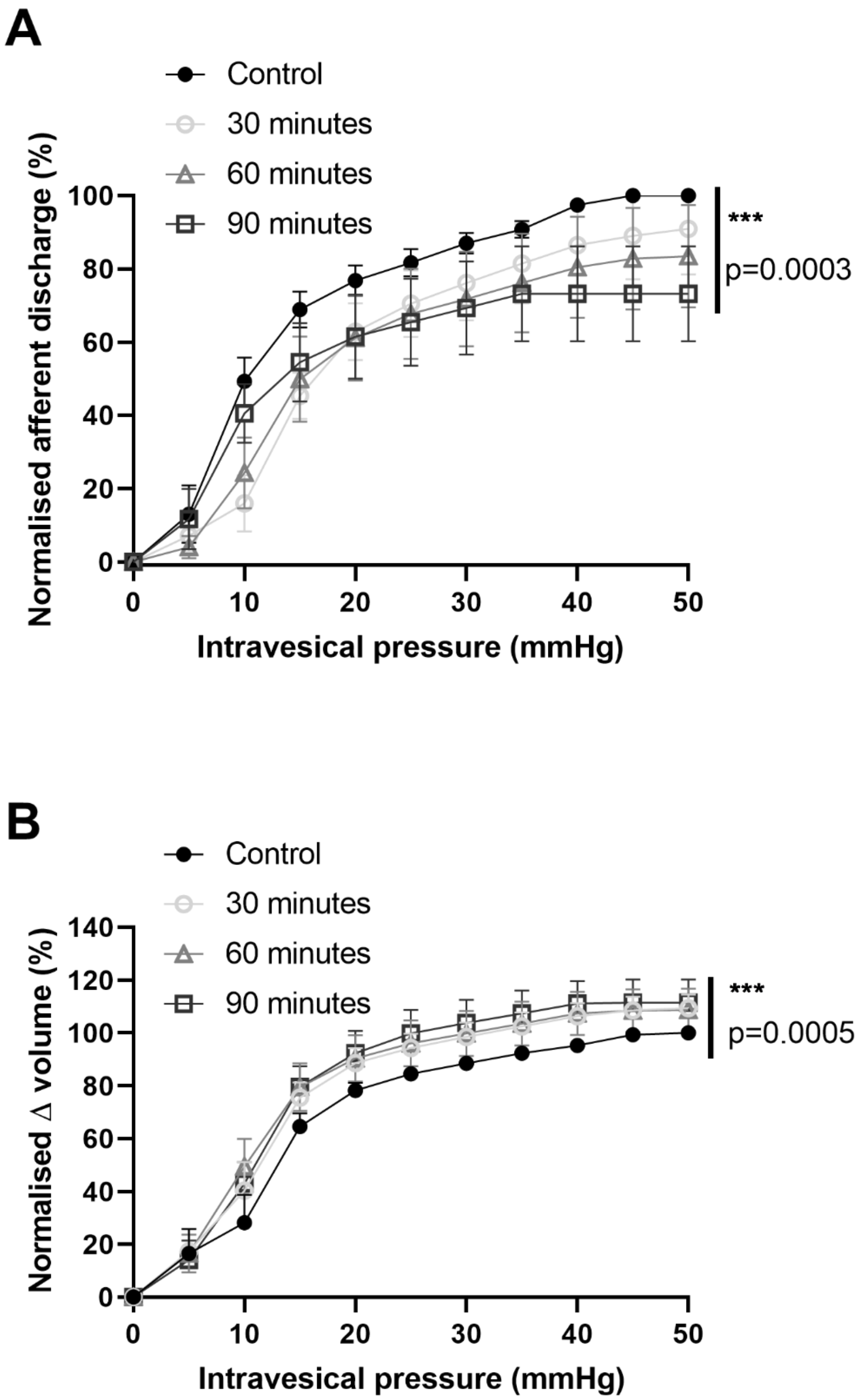


Figure 3.17: 3.6 pM LHn/A application led to significantly reduced bladder mechanosensation. **A)** Afferent responses to distension were significantly reduced 30, 60 and 90 minutes after treatment (p<0.0003, n = 7,

two-way ANOVA). **B)** the pressure-volume relationship was significantly increased after intravesical LHn/A compared to control (p = 0.0005; n = 7; two-way ANOVA)

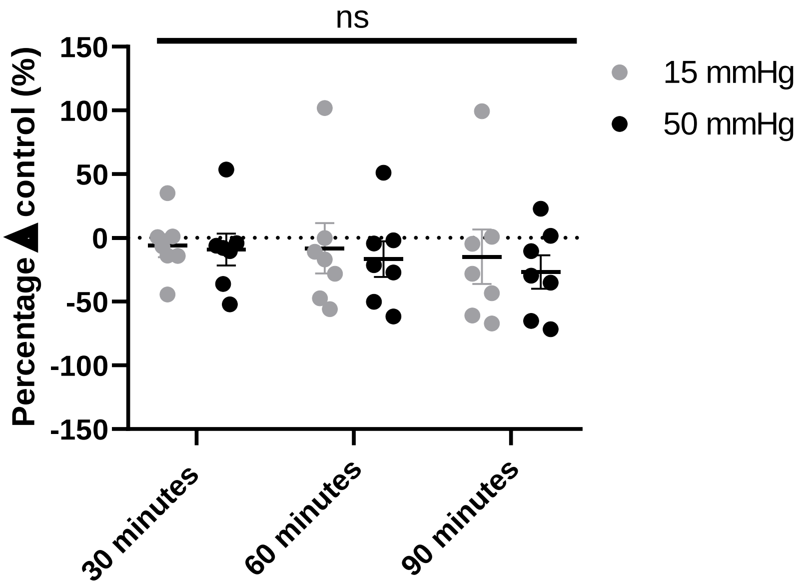


Figure 3.18: Percentage change of peak firing at 15 mmHg and 50 mmHg showed no significant difference between the early and later time points (p = 0.7375; *n* = 6; two-way ANOVA).

3.6 LHn/A dose dependently inhibits bladder mechanosensitivity

To understand whether LHn/A inhibited afferent firing in a dose dependent fashion, increasing concentrations were applied from 1 fM to 1 µM. LHn/A induced inhibition was more potent as the concentrations increased, with 1fM LHn/A having no effect on distension induced firing. In terms of the pressure-volume relationship, intravesical application of LHn/A appeared to have a dose dependent effect that was opposite to that of nerve firing. This is because lower concentrations increased compliance more than higher concentrations, with 1 fM significantly increasing compliance ($p < 0.0001$; $n = 3$) whereas 1 µM LHn/A had no effect ($p = 0.1975$; $n = 6$).

Effect of 1 fM LHn/A on bladder mechanosensitivity

Application of 1fM LHn/A did not alter bladder mechanosensitivity, as after 90 minutes post-treatment, 83.98% (+/-10.25%) of distension induced nerve firing remained ($p = 0.0992$; figure 3.19A; $n = 3$; two-way ANOVA). There was no time-dependent effect of 1 fM LHn/A on peak firing at 15 or 50 mmHg ($p = 0.0955$; figure 3.20). Interestingly, the pressure-volume relationship of the bladder was significantly increased by 1 fM LHn/A ($p < 0.0001$; figure 3.19B).

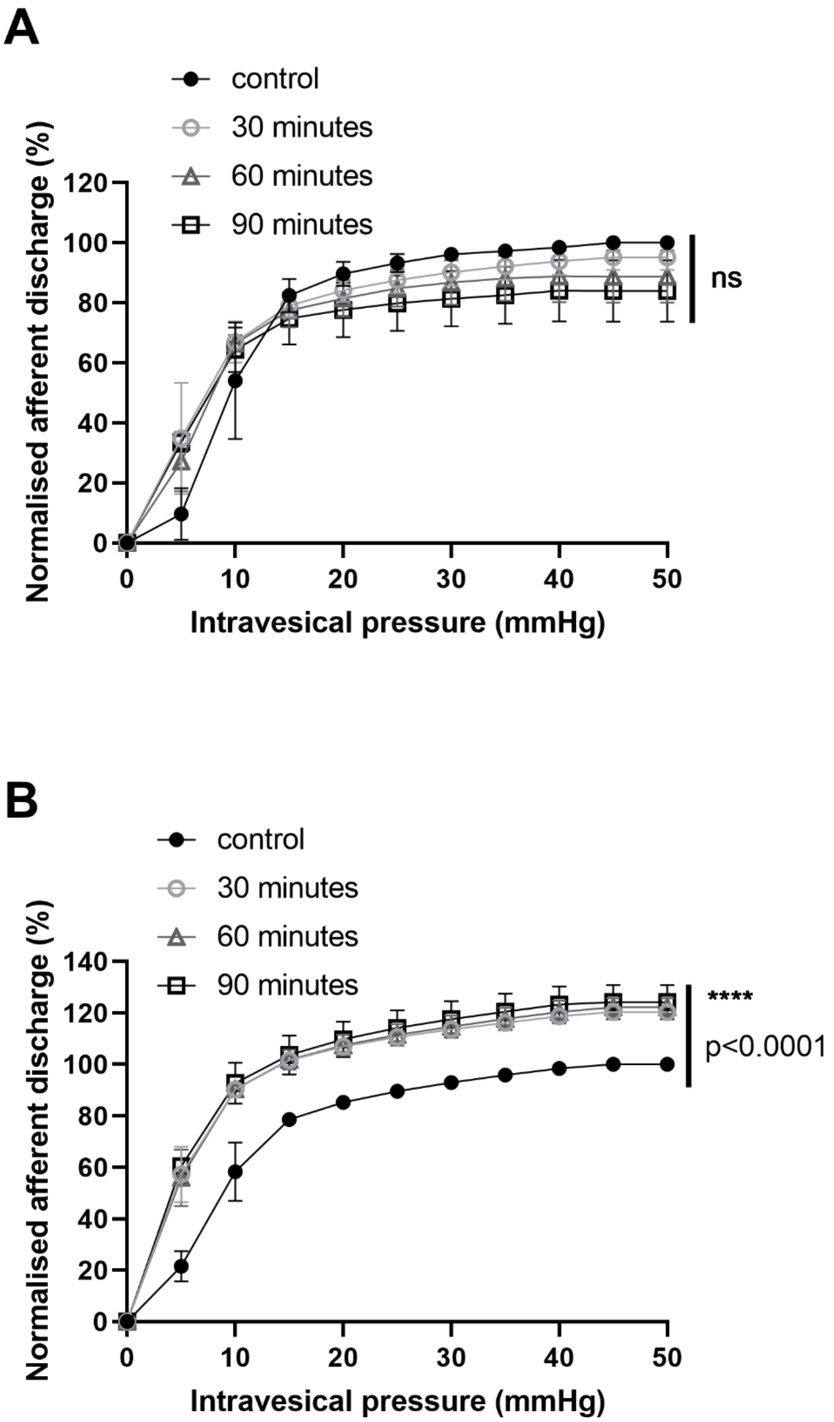


Figure 3.19: The effect of 1 fM LHn/A on bladder mechanosensitivity. **A)** Afferent responses to distension were unchanged 30, 60 and 90 minutes after treatment (p = 0.0992, *n* = 3, two-way ANOVA). **B)** the

pressure-volume relationship was significantly increased after intravesical 1 fM LHn/A compared to control (p = 0.0001; *n* = 3; two-way ANOVA).

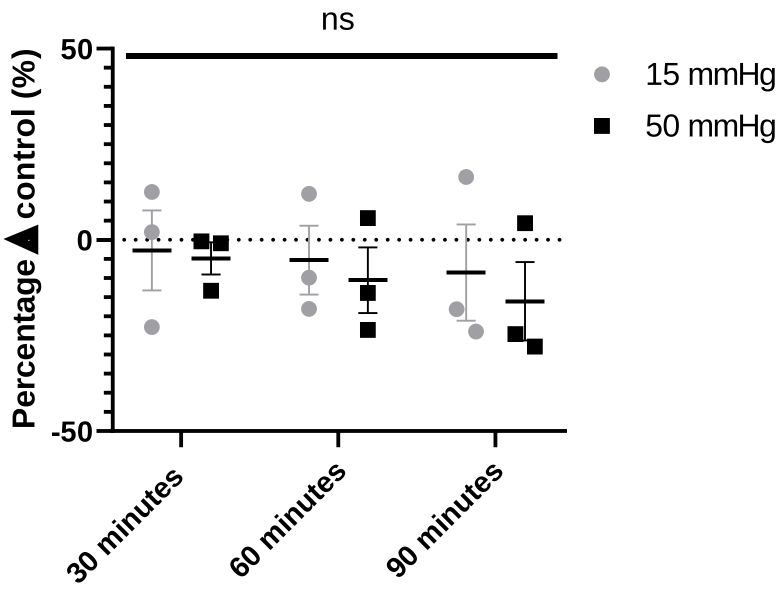


Figure 3.20: Percentage change of peak firing at 15 mmHg and 50 mmHg showed no significant difference between the early and later time points (p = 0.0955; *n* = 3; two-way ANOVA).

Effect of 300 nM LHn/A on bladder mechanosensitivity

Application of 300 nM LHn/A significantly reduced bladder mechanosensitivity, as after 90 minutes post-treatment, 68.6% (+/- 24.3%) of distension induced nerve firing remained ($p<0.0001$; figure 3.21A; *n* = 4; two-way ANOVA). Peak firing was not affected in a time dependent manner at 15 mmHg or 50 mmHg ($p = 0.1969$; figure 3.22; one-way ANOVA). The pressure-volume relationship was significantly increased following treatment with 300 nM LHn/A ($p = 0.0293$; figure 3.21B)

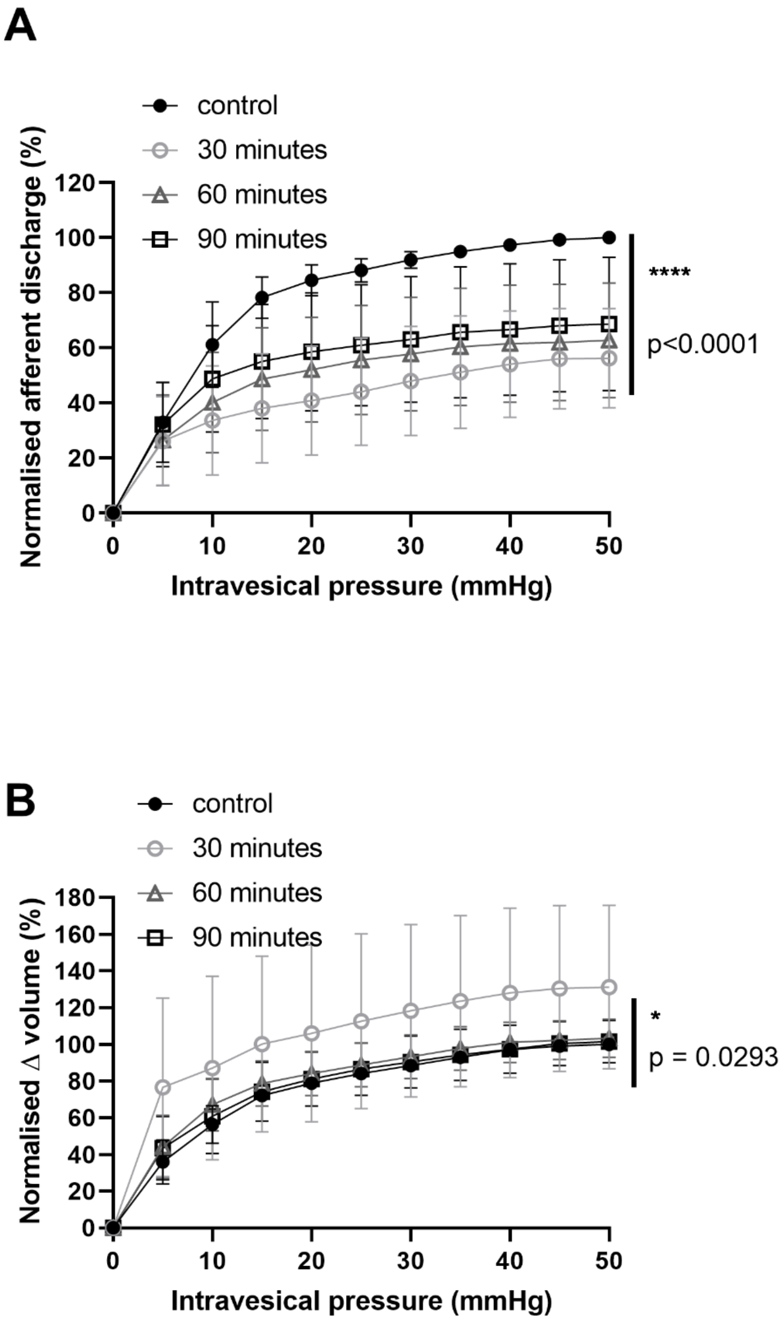


Figure 3.21: the effect of 300 nM LHn/A on bladder mechanosensitivity. **A)** Afferent responses to distension were significantly reduced 30, 60 and 90 minutes after treatment (p <0.0001, *n* = 4, two-way ANOVA). **B)** the pressure-volume relationship was significantly increased after intravesical 300 nM LHn/A compared to control (p = 0.0293; *n* = 4; two-way ANOVA)

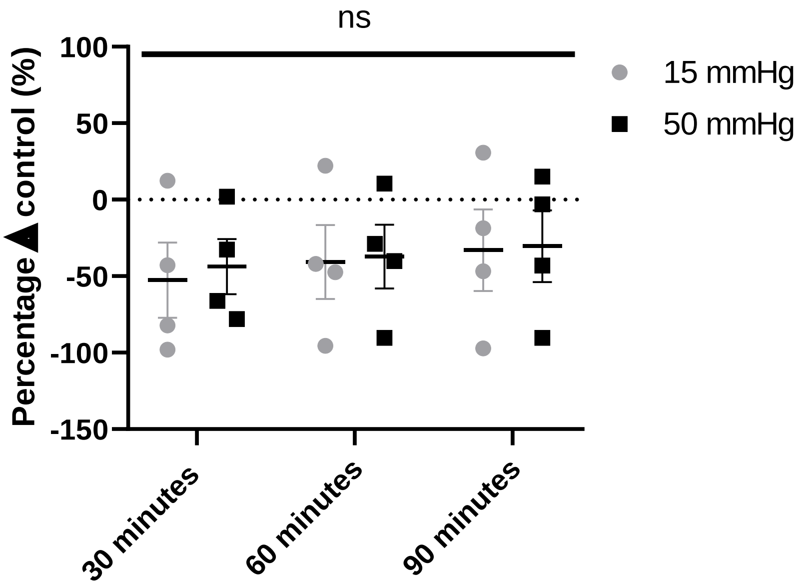


Figure 3.22: Percentage change of peak firing at 15 mmHg and 50 mmHg showed no significant difference between the early and later time points (p = 0.1969; *n* = 4; two-way ANOVA).

Effect of 1 µM LHn/A on bladder mechanosensitivity

Application of 1 µM LHn/A significantly reduced bladder mechanosensitivity, as after 90 minutes post-treatment, 54.69% (+/- 13.85%) of distension induced nerve firing remained ($p<0.0001$; figure 3.23A; *n* = 6; two-way ANOVA). Similarly to the other BoNT fragments tested in this chapter, the afferent inhibition induced by 1 µM LHn/A did not increase over time and did not differentially inhibit low threshold or high threshold fibres (p = 0.1183; figure 3.24). Pretreatment with 1 µM LHn/A did not appear to have an effect on the pressure volume relationship of the bladder, as there was no difference in the volume required to reach 50 mmHg over time (p = 0.1975; figure 3.23B).

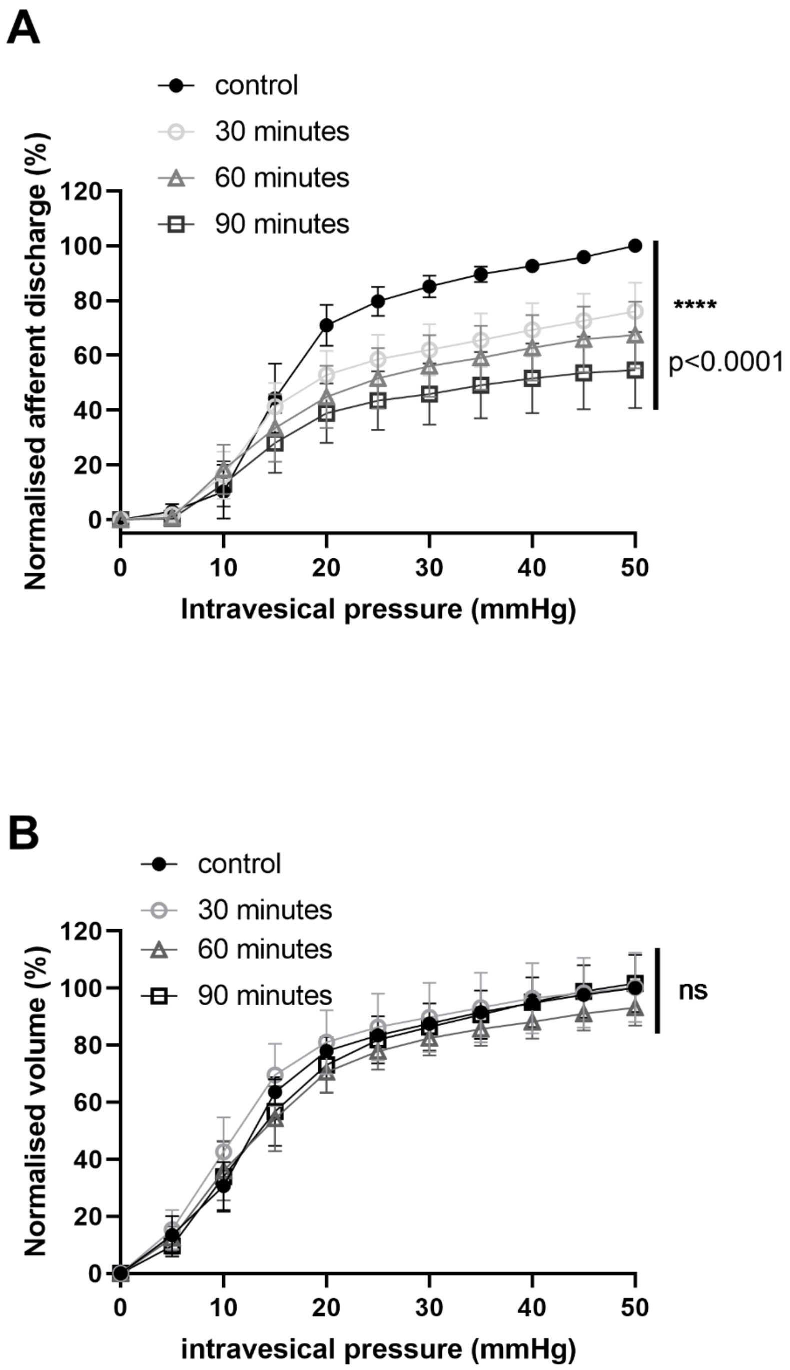


Figure 3.23: The effect of 1 µM LHn/A on bladder mechanosensitivity**. A)** Afferent responses to distension were significantly reduced 30, 60 and 90 minutes after treatment (p <0.0001, *n* = 6, two-way ANOVA). **B)** there appeared to be no difference in the pressure-volume relationship after intravesical 1 µM LHn/A compared to control (p = 0.1975; *n* = 6; two-way ANOVA)

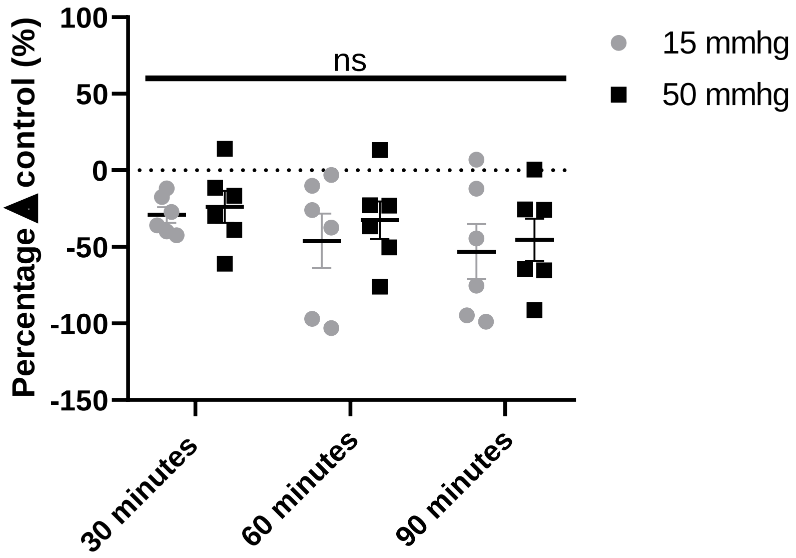


Figure 3.24: Percentage change of peak firing at 15 mmHg and 50 mmHg showed no significant difference between the early and later time points (p = 0.1183; *n* = 6; two-way ANOVA).

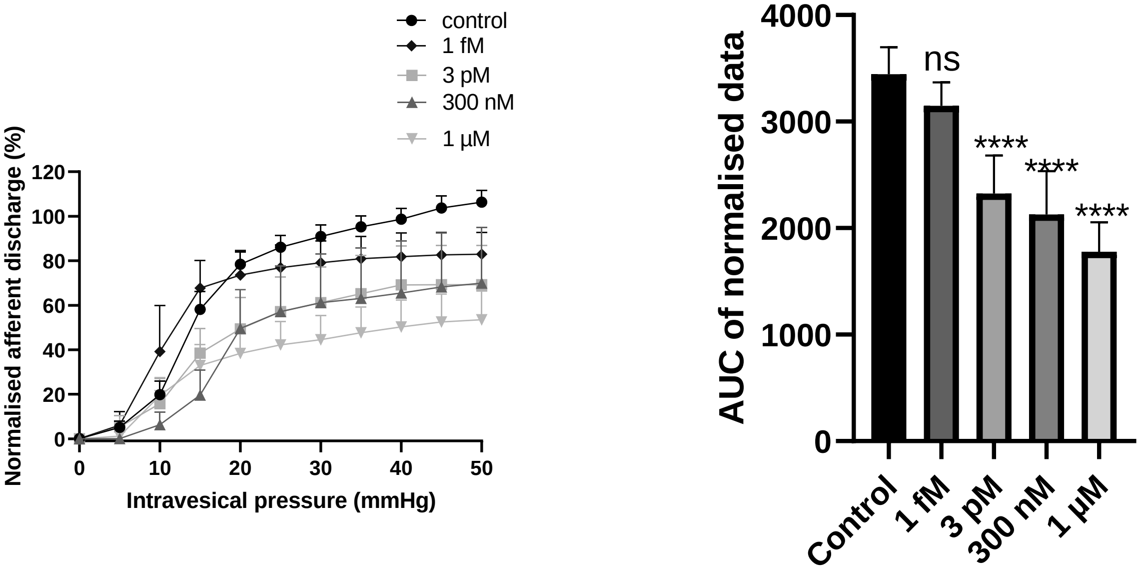


Figure 3.25: **A)** Afferent responses to distension significantly reduced 90 minutes after application of 1 fM, 3pM, 300nM and 1µM LHn/A (P <0.0001, 1 fM *n* = 3; 3 pM *n* = 5; 300 nM *n* = 4, 1µM *n* = 6). This data is also shown in **B)** as AUC, multiple comparisons of the AUC data shows significant reductions in afferent firing induced by 3 pM, 300 nM and 1µM (P <0.0001) but not 1fM (P>0.05). Analysed with one-way and two-way ANOVA with Bonferroni's post-test.

3.7 Effect of HC/A on bladder mechanosensation

To confirm the observed effect on bladder mechanosensation is due to activity induced by the light chain domain of BoNT/A, the effect of the heavy chain only (HC/A) domain was investigated next.

Application of 3.6 pM HC/A did not alter bladder mechanosensitivity (figure 3.25), as after 90 minutes post-treatment, 89.03% (+/-3.032%) of distension induced nerve firing remained (p= 0.9819; *n* = 4; figure 3.26A). The pressure volume relationship also remained unchanged 90 minutes following treatment with HC/A (p = 0.0513; *n* = 4; figure 3.26B). Peak firing at 15 and 50 mmHg did not appear to be altered over time (p = 0.0927; *n* = 4; figure 3.27)

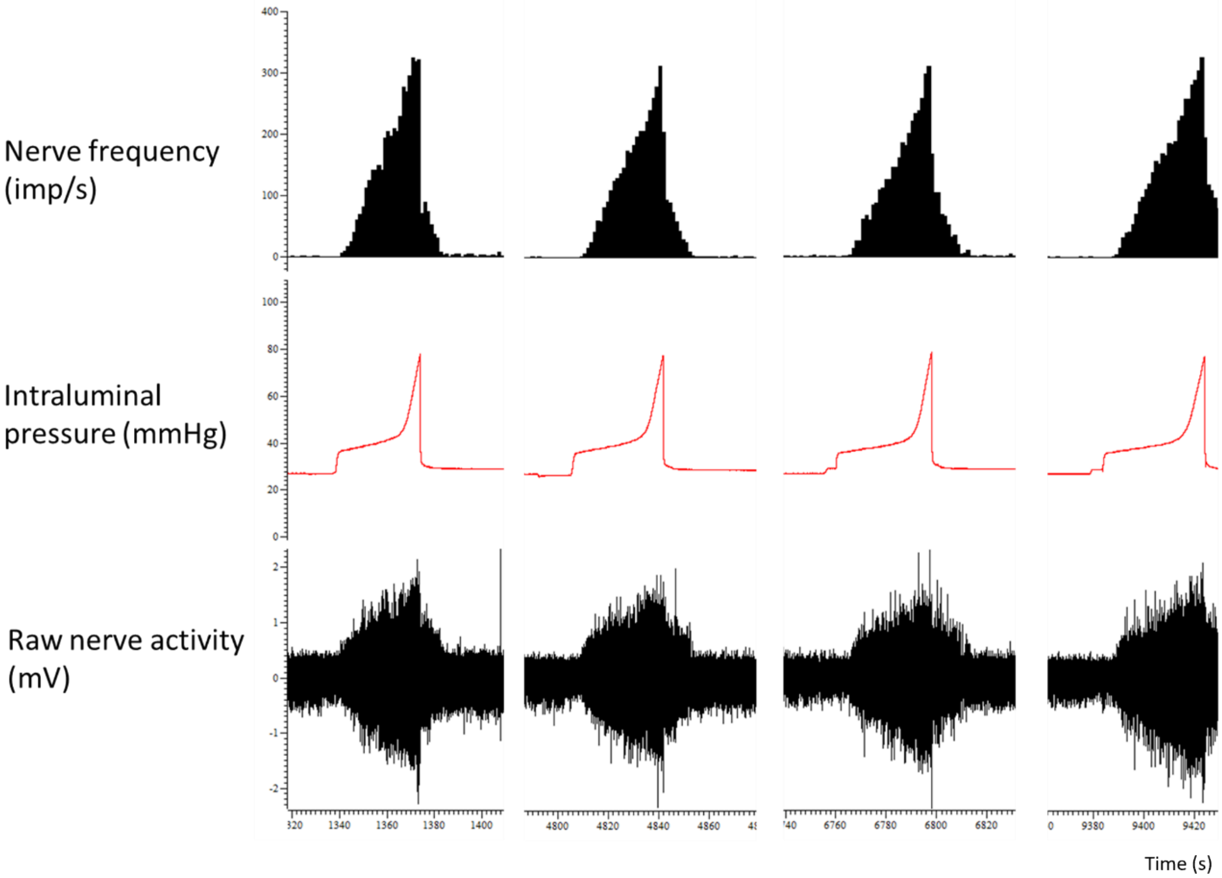


Figure 3.26: an example trace showing the response of afferent nerves to distension prior to (control) and 30, 60 and 90 minutes after application of HC/A.

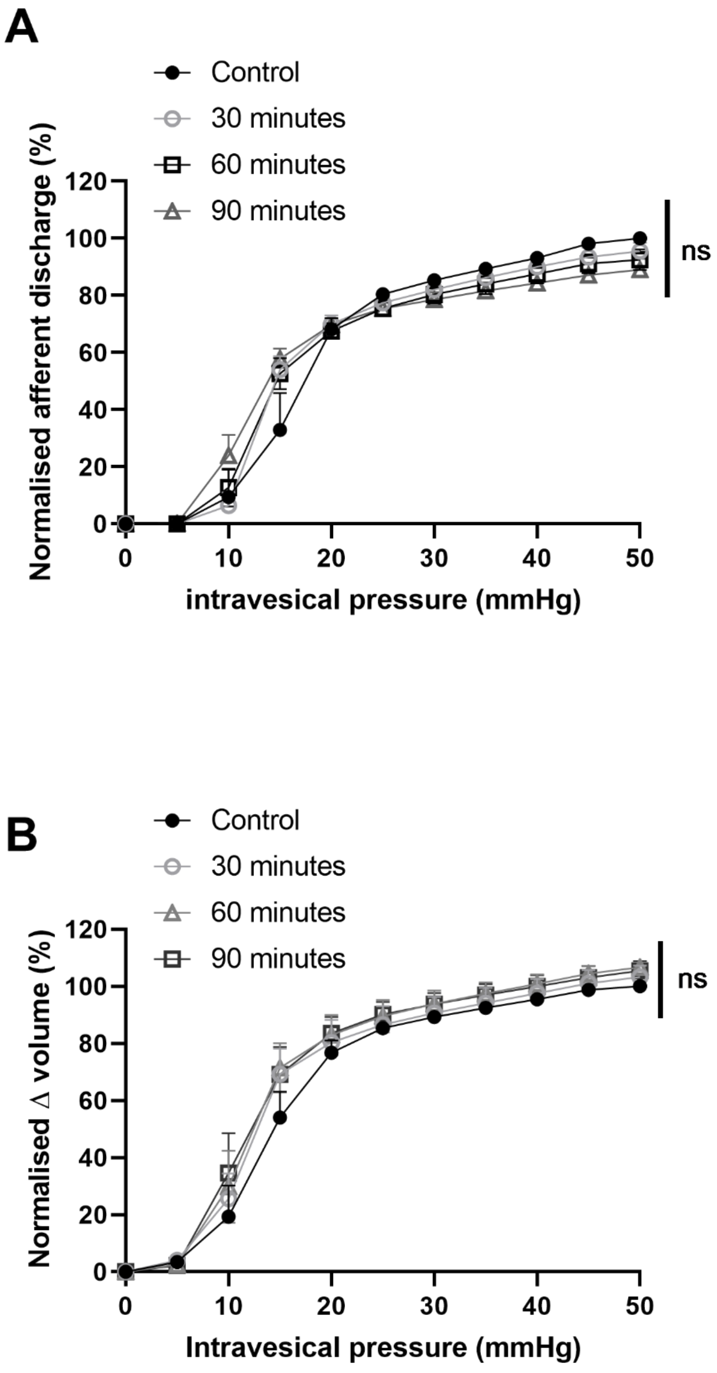


Figure 3.27: HC/A had no effect on distension induced firing. **A)** Afferent responses to distension were unchanged 30, 60 and 90 minutes after treatment (p = 0.9819, *n* = 4, two-way ANOVA. **B)** there appeared to be no difference in the pressure-volume relationship after intravesical HC/A compared to control (p = 0.0513; *n* = 4; two-way ANOVA).

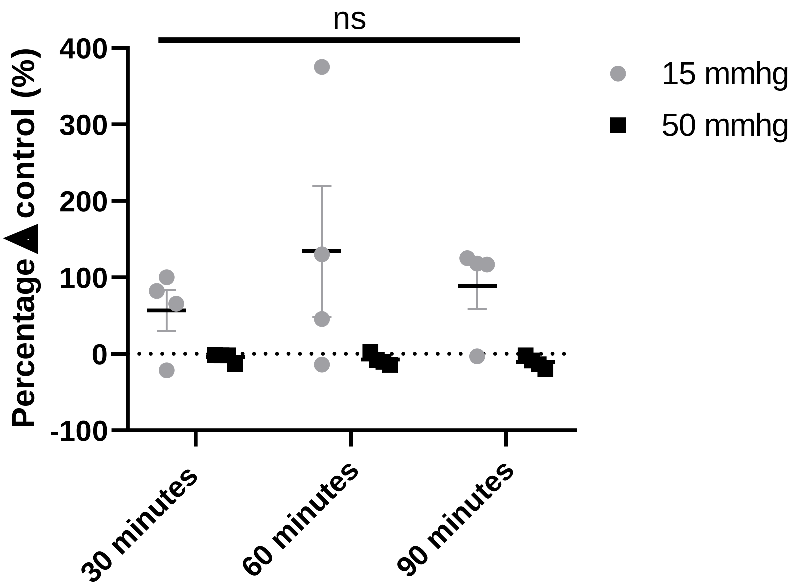


Figure 3.28: Percentage change of peak firing at 15 mmHg and 50 mmHg showed no significant difference between the early and later time points (p = 0.0927; *n* = 4; two-way ANOVA)

3.8 Effect of LC/A on bladder mechanosensitivity

To ensure the observed effect underlying the inhibition of sensory signalling in the bladder is due to the presence of the light chain, a LC/A only construct was used. Application of 3.6 pM LC/A significantly reduced bladder mechanosensitivity (figure 3.28), as after 90 minutes post-treatment, 71.36% (+/- 16.99%) of distension induced nerve firing remained ($p<0.0001$; figure 3.29A; *n* = 5). The compliance of the bladder was significantly increased by intravesical LC/A, which shows significantly more volume was required to reach 50 mmHg over the time course measured ($p<0.0001$; *n* = 5; figure 3.29B). Similarly to the other BoNT/A constructs discussed above, LC/A did not appear to differentially inhibit nerve populations responsive to physiological (15 mmHg) and nociceptive (50 mmHg) stimuli ($p = 0.2324$; figure 3.30).

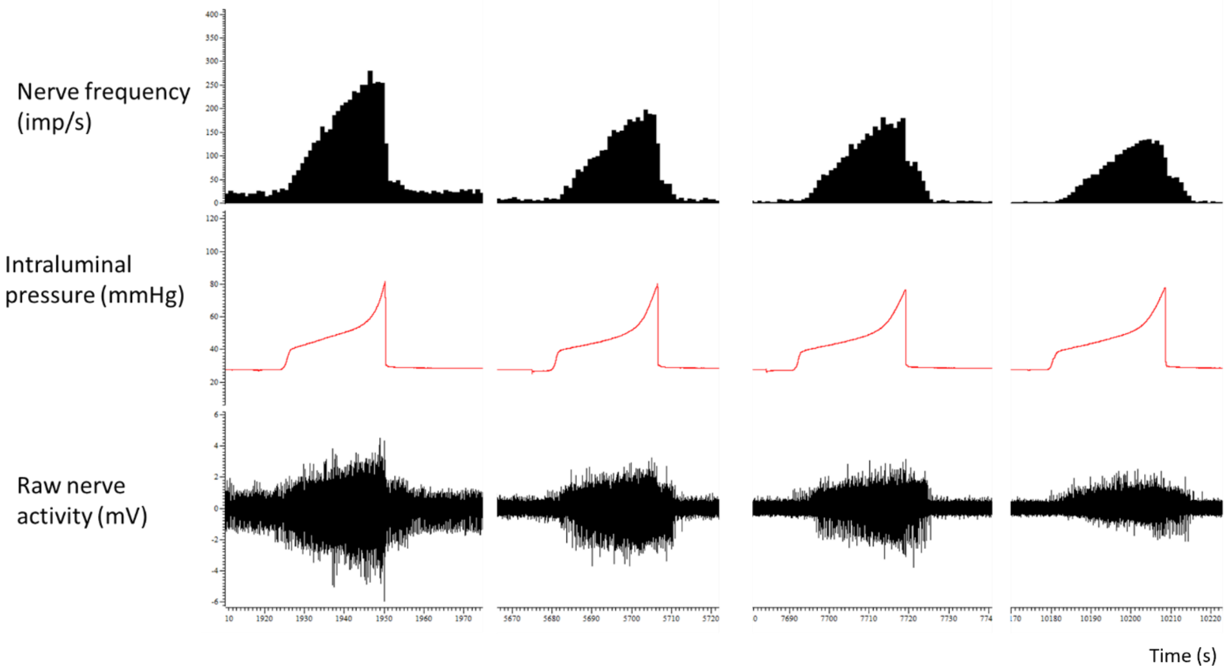


Figure 3.29: an example trace showing the response of afferent nerves to distension prior to (control) and 30, 60 and 90 minutes after application of LC/A

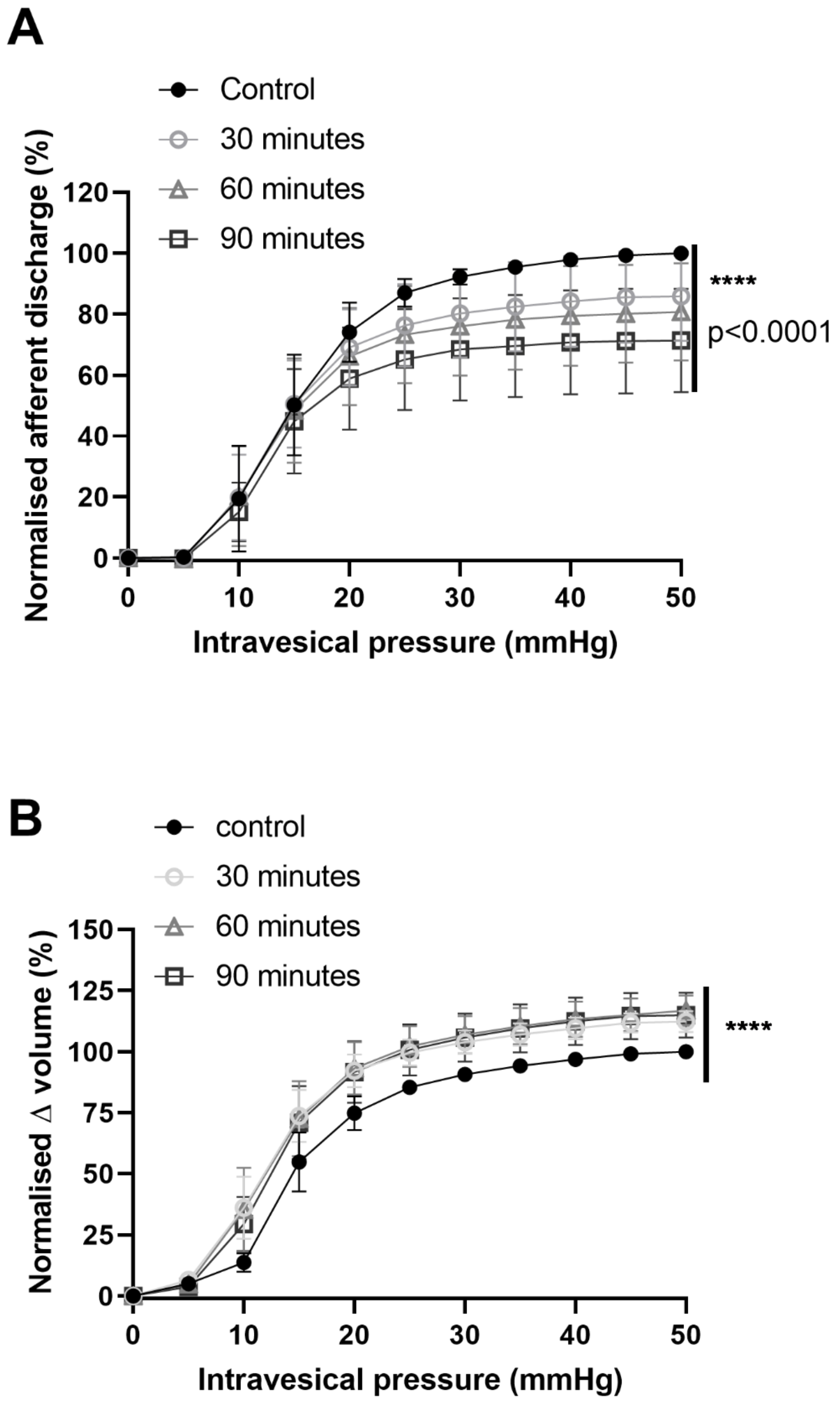


Figure 3.30: LC/A significantly inhibited bladder mechanosensitivity **A)** Afferent responses to distension were significantly reduced after intravesical LC/A treatment ($p<0.0001$, *n* = 5; two-way ANOVA). **B)** the

pressure-volume relationship was significantly increased following treatment (p = 0.0001; *n* = 5; two-way ANOVA).

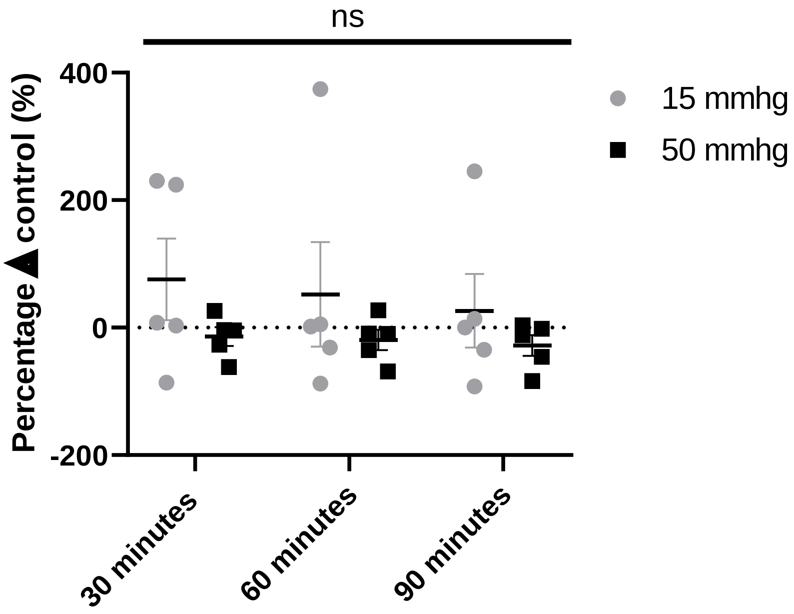


Figure 3.31: Percentage change of peak firing at 15 mmHg and 50 mmHg showed no significant difference between the early and later time points (p = 0.2324; *n* = 5; two-way ANOVA).

3.9 Engineering BoNT/A to target sensory nerves via modified receptor targeting region

Recent research in the BoNT/A field have concentrated on taking advantage of its ability to specifically target a particular cell type through its three-step receptor recognition, internalisation and SNARE cleavage mechanism. By replacing the receptor binding doman of BoNT/A with a moiety that binds to a specific receptor expressed on the cell type of interest, SNARE mediated exocytosis can be disrupted in a targeted manner. In this study, a targeted secretion inhibitor (TSI) engineered to bind to the TrkA receptor expressed on sensory nerve terminals, was tested to determine its effect on distension induced afferent firing.

Intravesical application of the TrkA-TSI at 300 nM did not have a significant effect on distension induced firing ($p = 0.2785$; $n = 4$; figure 3.31A). Peak firing at low and high threshold firing was unaffected following treatment with the TrkA-TSI ($p = 0.4384$; figure 3.32). Bladder compliance was significantly increased following treatment with the TrkA TSI ($p < 0.0001$; $n = 4$; figure 3.31B).

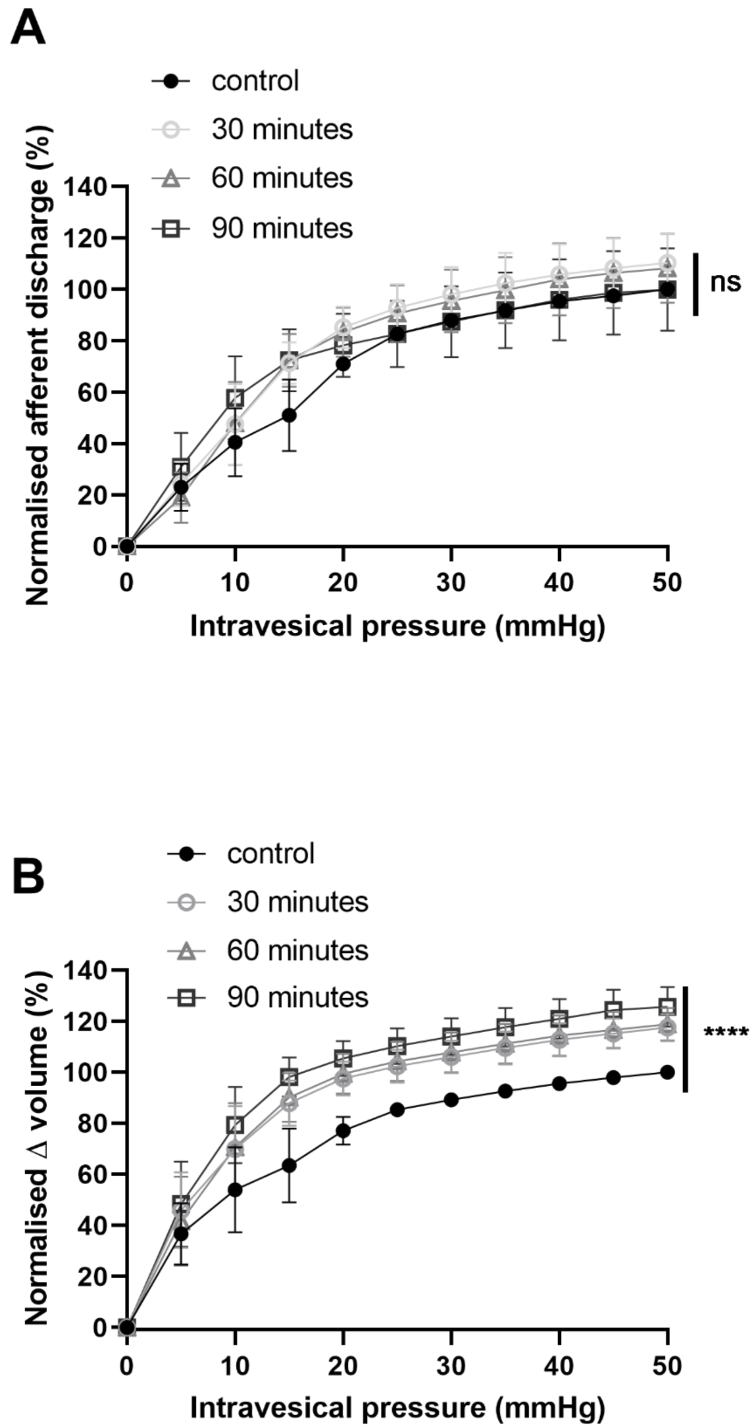


Figure 3.32: TrkA TSI did not affect bladder afferent firing. **A)** Afferent responses to distension were not altered after intravesical TrkA TSI treatment ($p = 0.2785$; $n = 4$; two-way ANOVA). **B)** the pressure-volume relationship was significantly increased following treatment ($p < 0.0001$; $n = 4$; two-way ANOVA).

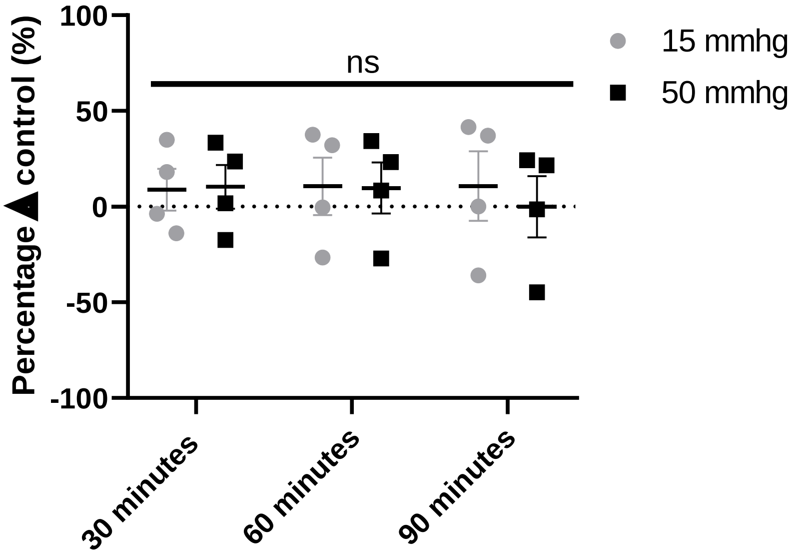


Figure 3.33: Percentage change of peak firing at 15 mmHg and 50 mmHg showed no significant difference between the early and later time points (p = 0.4384; *n* = 4; two-way ANOVA).

3.10 Presence of total and cleaved SNAP-25 in Dysport treated bladders

Due to the well-characterised classical mechanism of BoNT/A intoxication involving cleavage of SNAP-25, it was important to investigate whether the inhibitory effect observed in the nerve recordings was also due to this mechanism. The presence of total SNAP-25 in the bladder was investigated using an anti-SNAP-25 antibody (figure 3.33), immunoreactivity was found primarily in the suburothelial and detrusor regions, with little staining in the urothelial layer. The SNAP-25 immunoreactivity appeared to occur in the form of lines within the tissue, which could suggest staining was predominant in the suburothelial and intradetrusor nerve fibres.

Using an antibody targeted to the cleaved form of SNAP-25, the question of whether Dysport treatment led to cleavage of SNAP-25 was investigated (figure 3.34). Interestingly, there was no cSNAP-25 immunoreactivity in the Dysport treated bladders nor in the PBS (control) bladders. This contrasted with the immunoreactivity throughout the neuromuscular junctions of Dysport intoxicated rat skeletal muscle found in the positive controls shown in chapter 2.

These data suggest SNAP-25 is present in the bladder wall, however Dysport treatment in this preparation did not lead to immunoreactivity following treatment with cSNAP-25 antibody.

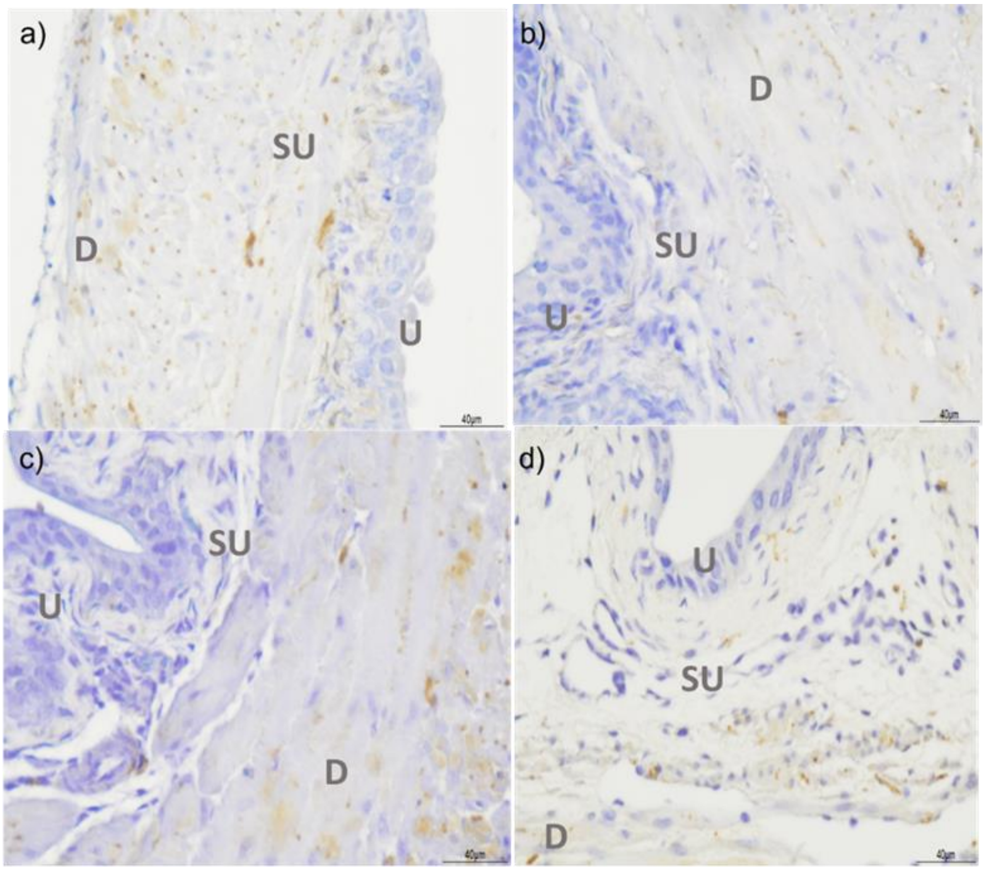


Figure 3.34: A representative figure showing PBS treated **(a)** and **(b)** and Dysport treated **(c)** and **(d)** bladder tissues labelled with anti-SNAP-25 antibody. SNAP-25 immunoreactive staining was found in the suburothelial and detrusor layers predominantly. Images labelled as U – urothelium, SU – suburothelium and D – detrusor.

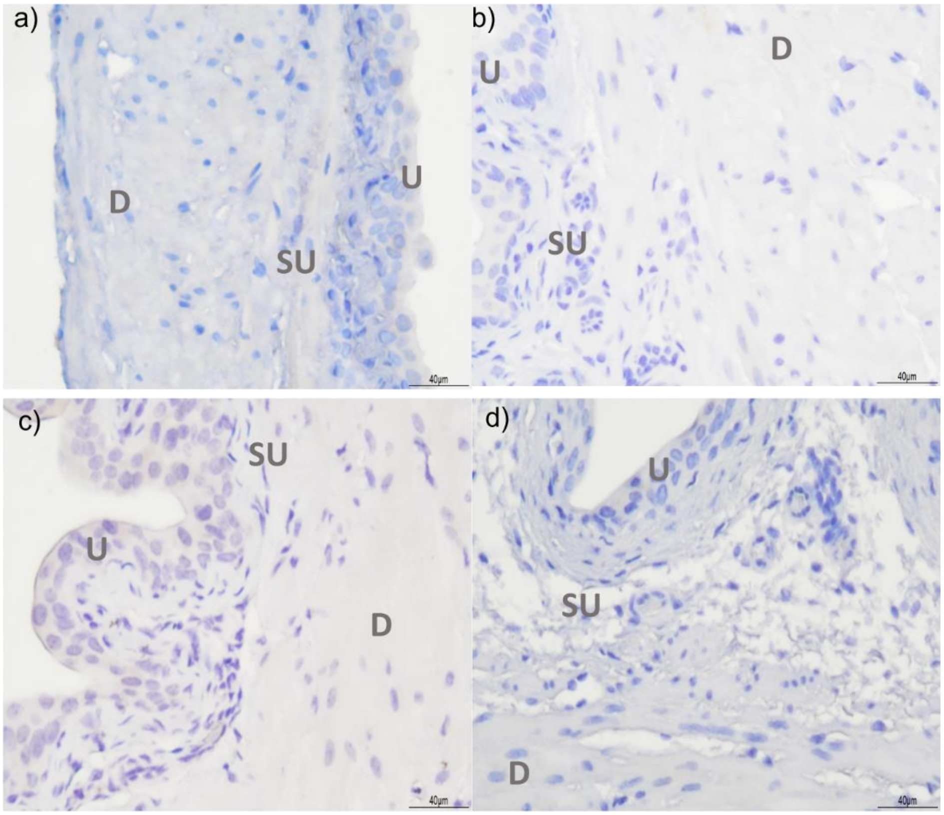


Figure 3.35: a representative figure of bladder sections labelled with anti cSNAP-25 antibody. PBS pretreated (a) and (b) did not show any positive staining, nor did Dysport pretreated bladders (c) and (d). images labelled as U – urothelium, SU – suburothelium and D – detrusor.

DISCUSSION

The main findings described in this chapter are

- Dysport, or abobotulinum neurotoxin A, significantly inhibited distension induced afferent firing from the mouse bladder.
- The non-toxic accessory proteins (NAPs) were not required for the BoNT/A induced inhibitory effect on bladder sensation, as the full-length toxin (nBoNT/A and rBoNT/A) alone exhibited similar effects to the BoNT/A complex.
- The SV2 binding region was not required for BoNT/A induced afferent inhibition, as LHn/A significantly inhibited afferent firing to a similar degree as full length BoNT/A.
- The HC domain alone did not have an effect on bladder sensation, while the LC domain inhibited bladder mechanosensitivity, suggesting the LC domain is important for the BoNT/A dependent observed effect.
- Application of the TrkA TSI molecule had no effect on distension induced firing.
- Dysport treated bladders did not exhibit cSNAP-25 immunoreactivity, although the full-length SNAP-25 was present in treated and untreated samples.

The aim of this chapter was to systematically characterise the effects of BoNT/A on bladder sensory signalling, and the contribution of its various subdomains and accessory proteins to this effect. Various formulations and production methods of BoNT/A were used to pinpoint the subdomains necessary to inhibit distension induced afferent signalling.

Dysport treatment significantly attenuated bladder mechanosensation

The symptom of urgency is central to the diagnosis of OAB, as the International Continence Society’s 2002 consensus definition of OAB describes it as urgency with or without urinary incontinence (Abrams et al., 2003). Previous clinical studies have reported that intravesical injection of Dysport improves symptoms of overactive bladder (OAB), resulting in reduced instances and severity of urge incontinence and increasing quality of life (Irwin et al., 2013; Kalsi et al., 2006; Manecksha et al., 2012; Pinto et al., 2010). While the effect of BoNT/A on bladder mechanosensitivity has been investigated previously using Botox (onabotulinum neurotoxin A; Collins et al., 2013), there was no data on whether Dysport acts directly on afferent mechanosensitivity. It is widely reported that Botox and Dysport are not completely bioequivalent due to differences in manufacturing (Field et al., 2018), which necessitated further investigation to clarify the effects of Dysport on afferent signalling. This study was conducted as an investigation of Dysport and its action on bladder sensation to improve understanding of its sensory effects in the clinic.

In this study, an *ex vivo* mouse bladder electrophysiology preparation was used, which is advantageous compared to *in vivo* preparations as it bypasses the confounding effects of CNS activity to facilitate targeted study of the visceral afferent system. This is especially useful in the study of BoNTs as it facilitates focused research of the direct effects on afferent nerve activity due to the lack of ongoing parasympathetic tone in the bladder. Following intravesical instillation of Dysport, significant inhibition of the distension induced activation of pelvic nerves was seen 90 minutes after exposure, as the raw nerve trace in figure 3.3 shows the loss of nerve units responsive to distension. These data show Dysport has direct effects on mechanosensitivity, like those previously reported using Botox (Onabot/A) (Collins et al., 2013).

The bladder is innervated by Aδ and C fibres which detect stretch and can be classified based on their conduction velocity. To understand the effect of BoNT/A on physiological and painful stimuli, the change in peak firing at 15 mmHg and 50 mmHg as compared to that of the control distension at the start of the experiment. The selected pressures were chosen as previous research has shown physiological pressures during micturition ranging between 5 – 15 mmHg, and painful pressures between 30 – 50 mmHg (de Groat & Yoshimura, 2009; Häbler et al., 1990), as mechanosensitive nerve fibres respond to different degrees of stretch within the bladder wall. By filling the bladder to a supraphysiological pressure such as 50 mmHg, the full spectrum of afferents, from low threshold to high threshold nociceptors within the nerve bundle can be stimulated and any effects of BoNTs on their activity can be measured.

Following exposure to Dysport, peak afferent firing at 15 mmHg was reduced to 42.5% (+/- 15%) of control and peak firing at 50 mmHg was reduced to 32.5% (10.7%). This was not significantly different ($p>0.05$; t-test), which suggests Dysport treatment may not differentially inhibit mechanosensitive nerves based on receptive threshold. This analysis does not define specifically which types of nerve fibre are affected by Dysport treatment, for example low or high threshold fibres, Aδ or C fibres, and peptidergic or non-peptidergic fibres. This information could be shown through conducting single unit analysis as previously described (Collins et al., 2013; Daly et al., 2007; Rong et al., 2002), or recording conduction velocity directly. However, in this study single unit analysis was not conducted since the recordings show a high degree of summation of spikes in response to distension, due to the high number of single units within recorded nerve bundles. This summation makes confident identification of individual single units difficult. The overall trend of data obtained in the pressure-nerve firing figures show there does not appear to be a distinct difference between the response profiles of low and high threshold responding nerves, suggesting that fibres were inhibited irrespective of their threshold. Conduction velocity is readily studied through *in vivo* recordings, however, there are drawbacks to *in vivo* experiments that may have affected interpretation of results, for example active CNS innervation and potential effects of BoNTs on efferent signalling. For these reasons, *ex vivo* recordings were chosen for this thesis. Identifying the properties and subtypes of the fibres affected by Dysport would be an interesting avenue for research and worth considering in future studies.

The best described mechanism of action of BoNT/A function is the intracellular cleavage of the SNARE protein SNAP-25 at the neuromuscular junction which results in flaccid paralysis (Blasi et al., 1993). To investigate whether the inhibitory effect on bladder afferent firing was achieved by SNARE cleavage, an immunohistochemical study was conducted using antibodies that target SNAP-25 and its cleaved form (figures 3.33 and 3.34). The antibody used to visualise presence of cleaved SNAP-25 was generated in-house, and a positive control experiment was conducted to ensure it was able to recognise presence of cSNAP-25. Rat skeletal muscle that had received Dysport injection showed dense staining in the neuromuscular junctions and little to no staining in the myocytes (see chapter 2).

Due to the lack of cSNAP-25 immunoreactivity in the bladders that received intravesical instillation of Dysport, this data suggests that SNAP-25 may not be responsible for causing the inhibition of afferent nerve signalling. A limitation of this finding is the reliance of an in-house antibody, as previous authors have found variable specificity when testing their in-house produced antibody in a variety of tissue types (Rhéaume et al., 2015). It is encouraging that the cSNAP-25 antibody did not show non-specific staining in the vehicle treated bladders, however, replication of this finding using another cSNAP-25 targeting antibody would increase confidence in these results.

The lack of cSNAP-25 immunoreactivity may not be indicative of Dysport working through means outside of the classical mechanism and could instead be due to the method of administration. Coelho *et al* (2012) found significant neuronal staining following intramural injection of Botox, but not intravesical instillation as was conducted in this study (Coelho et al., 2012). There is also the suggestion that Dysport may have cleaved SNAP-25 in the present preparation, but that it was been below the threshold of detection of this antibody. However, the role of SNAP-25 cleavage on BoNT/A induced inhibition of bladder sensation was investigated in depth later in this thesis in chapter five and the conclusions from that work lead to the speculation that Dysport inhibits afferent firing from the bladder in a mechanism which is independent from cleavage of SNAP-25. This will be discussed at length later in the thesis.

BoNT/A can inhibit afferent mechanosensitivity without the non-toxic accessory proteins

One of the aims of this study was to further understand BoNT/A's mechanism of entry in the bladder. BoNT/A is secreted from *C. botulinum* as a multimeric complex, along with haeamagglutinins and non-toxic non-haemagglutinin (NTNH) proteins, known collectively as the non-toxin accessory proteins (NAPs). The function of the NAPs appears to be the enhancing of BoNT/A toxicity by protecting it from damage through the harsh environments of the gastrointestinal tract (Ohishi et al., 1977). This could be due to the pH dependency of the BoNT/A – NAP association, as the complex is stable at low pH but rapidly dissociates at pH 7 or higher (Eisele et al., 2011). The NAPs have also been implicated in the insertion of BoNT/A across colonic plasma membranes in the development of botulism (Kwangkook, 2014). This has been shown to occur through the disruption of the epithelial barriers, Kwangkook et al (2014) showed that BoNT/A haemagglutinins bind to cell adhesion protein e-cadherin, and when they impaired the haemagglutinins carbohydrate binding action, the oral toxicity of BoNT/A was reduced (Kwangkook, 2014). These data suggest *C. botulinum* has made considerable adaptations specific to the mammalian physiology, as increased lethality of the BoNT/A complex provides a new anaerobic environment in which to proliferate.

Previous preclinical studies have shown the NAPs increase the internalisation of BoNT/A which may improve its efficacy (Ghosal et al., 2018). To better understand whether presence of NAPs leads to an improved inhibitory effect of distension induced afferent firing, two 150 kDa neurotoxin only formulations were tested. A purified *C. botulinum* derived native BoNT/A, or nBoNT/A, and a recombinant BoNT/A, or rBoNT/A, were compared to Dysport. Interestingly, the three forms of BoNT/A had almost identical effects on distension induced afferent firing (figure 3.15; $p = 0.4507$), which suggests the presence of NAPs did not contribute to the sensory effects of BoNT/A. This suggests that the mechanism described in colonic cells may not translate to the bladder. Although it is important to note, the mammalian urothelium has been shown to have a very steep concentration gradient that maintains an efficient barrier, to stop the absorption of urine into the blood (Negrete, 1996), whereas one of the main functions of the colonic epithelium is absorption. The structural and functional differences between the two epithelial layers may explain why the NAPs may not have been as effective in facilitating BoNT/A insertion into the bladder.

It is unclear whether the NAPs confer an advantage to BoNT/A in the clinical context. In their study on the dissociation of the BoNT/A complex, Eisele *et al* (2011) report that upon reconstitution with saline, both Botox and Dysport dissociate rapidly within a minute with almost no complexes remaining (Eisele et al., 2011). They suggest this dissociation occurs too fast to have any meaningful effect on the therapeutic mechanism of BoNT/A, which could explain the lack of improvement of the BoNT/A mediated sensory inhibition found in the present study. This view is supported by a randomised clinical trial that showed no difference in the therapeutic effect of Botox and Xeomin, a 150 kDa formulation of BoNT/A that contains no NAPs, in patients with neurogenic detrusor overactivity (Giannantoni et al., 2021).

The light chain was the only domain necessary to induce the inhibitory effect on distension induced afferent firing

Through a systematic assessment of the BoNT/A molecule using recombinant subdomains, it was possible to define the domain necessary to inhibit bladder sensation. The subdomains were defined based on their role in inducing neuromuscular paralysis, the heavy chain responsible for receptor binding and translocation, while the catalytic light chain cleaves SNAP-25. Although previous research has shown BoNT/A has direct effects on sensory signalling, what roles the subdomains play in facilitating this response and whether they correspond to the role played in silencing cholinergic neurons is unclear. To answer this question, recombinant protein methods were utilised to produce the HC/A and the LC/A domains, as well as a construct missing the SV2 binding domain named LHn/A. These proteins were subsequently assessed to reveal any effects on bladder mechanosensitivity.

The first question tackled in this thesis was regarding the necessity of the SV2 binding domain on the BoNT/A induced afferent inhibition. The receptor dependency of BoNT/A was shown by Dolly *et al* (1984), who found internalisation and inhibition of neurotransmitter release induced by BoNT/A depended on receptor binding that was specific to cholinergic neurons, and not seen in other cell types (Dolly et al., 1984). The identity of the receptor was revealed by Dong *et al* (2006), who showed BoNT/A was unable to enter neuronal cells which did not express SV2 (Dong et al., 2006). Although the identity of the BoNT/A receptor was unknown before this, knowledge of its unique binding specificity and the location of the receptor binding region on the BoNT/A protein led to the idea that it could be removed and replaced with another targeting ligand, to retarget the SNAP-25 cleaving light chain to a wider group of cell types. Chaddock *et al* (2000) removed the cell binding domain (H_C domain) of BoNT/A to create LHn/A, a molecule that is identical to the full length BoNT/A except for the absence of the H_C region (Chaddock et al., 2000a). The receptor dependency of the BoNT/A molecule was exemplified by the inability of the LHn/A molecule to inhibit release of neurotransmitters from three cell types, PC-12, SH-SY5Y and embryonic spinal cord neurons (Chaddock et al., 2000a). This was contrast to the effect of LHn/A conjugated with wheat germ agglutinin (WGA), a lectin found in plasma membranes (Gabor et al., 1998), which significantly inhibited neurotransmitter release (Chaddock et al., 2000a). These data suggest a receptor, whether the native H_C region, or a conjugated ligand, is essential for the cell entry and catalytic activity of BoNT/A.

Although the effect of BoNT/A on bladder sensation has been investigated previously, its mechanism of entry into the bladder remains unclear. The expression of the SV2 receptor has been found on the urothelial cells, suburothelial and intradetrusor nerve fibres (Coelho et al., 2010; Giannantoni et al., 2011; Hanna-Mitchell et al., 2015; Rahnama'i et al., 2013). The question of whether BoNT/A may inhibit sensory afferent firing without binding to SV2 has not been answered previously and was investigated in this chapter through the use of LHn/A. Intravesical LHn/A significantly inhibited distension induced nerve firing at 3 pM, the same toxin concentration as previously shown in the full length BoNT/A (figure 3.17; p=0.0003). LHn/A induced inhibition occurred in a concentration dependent manner. This data stands in conflict with those discussed above, showing LHn/A is unable to enter cells without a conjugated ligand that corresponds to a residue on the cell surface. Further analysis showed there was no significant difference between full length BoNT/A constructs and LHn/A, which suggests the SV2 binding region was not involved in the inhibition of afferent signalling. It is important to note that the translocation domain, or Hn domain, is present on the LHn/A molecule, which suggests that if LHn/A was somehow endocytosed into the cell, it is still able to translocate into the cytosol and reach SNAP-25. The relative importance of the Hn domain in the induction of afferent inhibition is discussed in further detail below.

In order to further characterise the BoNT/A molecule, the light chain and heavy chain domains were produced and investigated separately. Interestingly, the LC/A domain alone was able to induce afferent inhibition, despite the lack of the receptor and translocation domains (figure 3.29; p<0.0001). This data confirms that the light chain is the source of the afferent inhibition, however it is still unclear how the LC/A protein is able to enter the bladder wall, how it reaches its cells of interest, and which cells those are. The first cells encountered by the LC/A protein once intravesically instilled into the bladder are the urothelial cells, which play a role in maintaining bladder mechanosensation through the release of neurotransmitters such as ATP (Ferguson et al., 1997). Previous research has shown BoNT/A treatment reduces ATP release from urothelial cells, which has been hypothesised to occur through the cleavage of SNAP-25 (Collins et al., 2013; Hanna-Mitchell et al., 2015; Khera et al., 2004; Nakagomi et al., 2016; Smith et al., 2005, 2008). There is a possibility that the LHn/A molecule may enter urothelial cells without the SV2 binding domain by taking advantage of the endocytic mechanisms of the umbrella cells following bladder emptying. This entry route may also be taken by the LC/A protein, however, it is less clear how it may escape the endocyte and enter the cytoplasm without the pore created within the endocyte membrane by the H_N translocation domain. Further studies are necessary to understand the movement of the LC/A protein to reach its target and whether it is still able to recognise and bind to SNAP-25.

The HC/A domain did not have any effect on bladder sensation, which supported the hypothesis of the receptor binding region not playing a role in the inhibition of distension induced firing (figure 3.26; $p = 0.9819$). This finding supports the idea that the LC/A domain is solely responsible for the action of BoNT/A on sensory signalling.

Retargeting BoNT/A to TrkA expressing cells by changing the receptor binding domain did not affect distension induced afferent firing

The targeted secretion inhibitor (TSI) technology was developed as a method of engineering therapeutics based on the BoNT backbone. The modular nature of BoNTs facilitates the replacement of the original SV2 binding region with a ligand of choice, targeting specific cell types in order to inhibit vesicular release mechanisms.

In this study, a TSI molecule that contained a binding ligand targeting the tropomyosin receptor kinase A (TrkA) receptor was tested. TrkA receptors expressed on sensory nerve terminals are activated by nerve growth factor (NGF) to trigger signal transduction pathways resulting in pain and hyperalgesia (Bergmann et al., 1998). Once activated, the NGF-TrkA complex has been shown to be endocytosed, which provides the acidified endosome required to induce conformational changes in the Hn domain to create the pore and release the light chain (Grimes et al., 1996). The TrkA receptor was intended to be used as a marker for nociceptive neurons, and target SNAP-25 cleavage to those neurons in order to halt sensory signalling and release of neuropeptides.

In this preparation, the TrkA-TSI was applied intravesically in the same method as the other BoNT/A constructs described in this chapter, however, there were no changes in the response to distension induced afferent firing (fig 3.32; p = 0.2785). While the TrkA-TSI was produced with the intention of showing a more pronounced effect on sensory signalling than the unliganded LHn/A and LC/A proteins, it instead had no effect. It is unclear why adding a ligand to the LHn/A backbone would lead to a loss in the inhibitory activity of the molecule, perhaps this is due to structural changes as the added size of the TrkA targeting antibody could have made it more difficult to enter through the usual mechanism. Also, the basal expression of TrkA in the bladder wall may not have been at an adequate level for the TSI molecule to bind to. Previous research has shown significant increases in TrkA expression in the bladder and DRG following chronic spinal cord injury and inflammatory conditions such as cyclophosphamide induced cystitis (Coelho et al., 2015; Murray et al., 2004; Qiao & Vizzard, 2002). It is possible that the TrkA TSI molecule may have more utility in inflammatory disorders where there is increased NGF activity and TrkA expression, which could be shown experimentally by conducting experiments using animal models of chronic pain.

Effects of BoNTs on bladder compliance

The ability of the bladder to store and eliminate large volumes of urine over a lifetime is dependent on the mechanical properties of the bladder wall, which create a balance between elasticity and stiffness. The extracellular matrix made up of collagen and elastin, as well as smooth muscle cells within the bladder wall facilitate the elasticity to accommodate large volumes of urine, while maintaining a level of stiffness to prevent overdistension (Murakumo et al., 1995; Parekh et al., 2010). In *ex vivo* preparations, the loss of ongoing parasympathetic tone to the detrusor means that the compliance measured is solely a result of the structural components. Hornsby et al (2017) conducted a study on the biomechanical properties of the bladder wall in an *ex vivo* preparation, finding that the deformation of the bladder wall during stretch dependent on the unfolding of collagen fibrils within the lamina propria and detrusor (Hornsby et al., 2017).

In the *ex vivo* bladder electrophysiology preparation, a readily available analysis is the calculation of the pressure volume relationship, or the compliance. In previous *ex vivo* studies, bladder compliance was defined as a measure of the accommodation of the volume within the bladder as it relates to intravesical pressure (Daly et al., 2007; Rong et al., 2002). This analysis was conducted in the present study, finding that the BoNTs tested in this preparation appeared to have differential effects on the pressure-volume relationship, with some having no effect and others showing significant increases. However, significantly increased compliance was also seen in the time control preparations which had been treated with PBS only. This suggests the differences may not be due to the action of the BoNTs, but a characteristic of the preparation, as bladder distensions to a supraphysiological pressure (50 mmHg) was performed once every ten minutes for upwards of two and a half to three hours. This also reveals a flaw in the pressure-volume analysis, while it gives a general idea about the capacity and mechanical responsiveness of the bladder, it is not sensitive enough to inform interpretation of what happens to the structure of the bladder wall to produce changes in capacity. Interestingly, the stability of the afferent nerve responses which show reproducibility throughout the experimental period in the time control recordings suggest that this was not affected in a similar way. These questions may be clarified in further studies where the stress and strain imposed to the bladder wall is measured directly as previously described (Saxena et al., 2023). This may allow us to understand whether BoNTs may interact with the structural components of the bladder wall, as well as how increased elasticity of the bladder wall may affect the mechanosensitivity of the afferent nerves

CONCLUSION

The results in this chapter provide evidence supporting the literature describing the effects of BoNT/A on bladder sensation, as well as evidence disputing the mechanism of entry of BoNT/A and its subdomains into the cells of interest. Although extensive literature describes the essential role of the SV2 binding region for recognition of target cells, LHn/A and the light chain only LC/A induced significant inhibition of bladder mechanosensation. This was not seen following intravesical treatment with the heavy chain, which supports the hypothesis that the subdomain of interest is the light chain. The role of SNAP-25 cleavage in the induction of afferent inhibition was also put into question in this chapter, as there was no cleaved SNAP-25 protein visible despite the extensive immunoreactivity of the full-length SNAP-25 in the suburothelial and intradetrusor nerve fibres. These results point to an alternative mechanism of entry and action of BoNT/A, which deserve further study to continue the development and expansion of its uses in the clinic.

CHAPTER 4 – THE EFFECTS OF BONTS /B AND /E ON BLADDER MECHANOSENSITIVITY

INTRODUCTION

The BoNT family includes seven distinct neurotoxins classified based on serotyping (/A to /X), all of which target the proteins making up the SNARE machinery governing regulated exocytosis to silence neurotransmission. BoNT/A is the best-characterised member of the BoNT family, both at the bench and in the clinic, and has achieved remarkable success in the treatment of various neuromuscular and sensory disorders. In the previous chapter, an in-depth analysis of BoNT/A entry and action in the bladder was conducted, which focused on its role in inhibiting sensory signalling. To continue this exploration of BoNT action on bladder sensation, two more members of the BoNT family, BoNTs /B and /E, were investigated

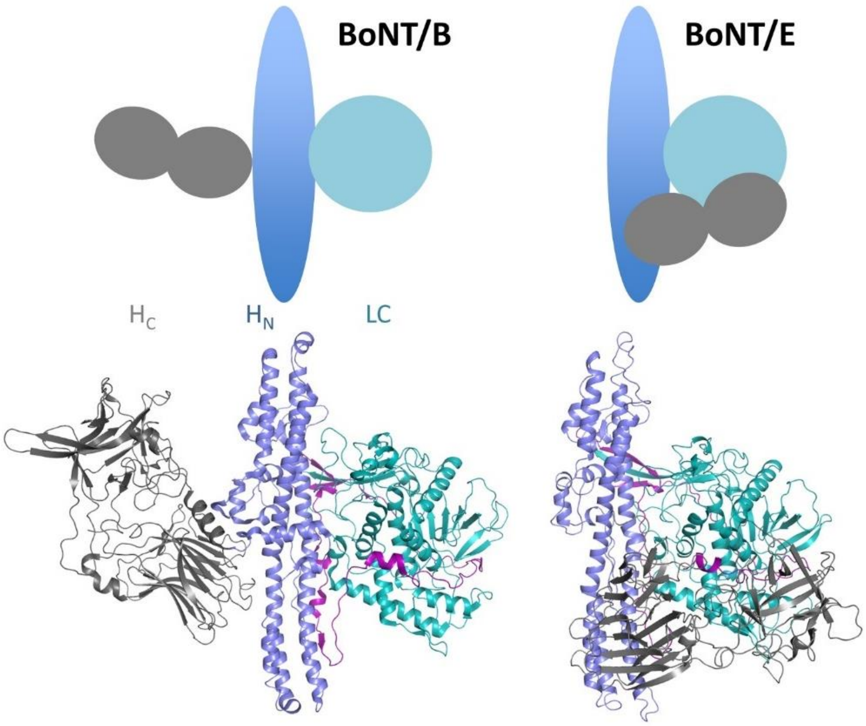


Figure 4.1: Structure of BoNTs /B and /E. The upper panels show the organisation of the subdomains within each BoNT, with the H_C domain in grey, the H_N domain in blue and the LC domain in cyan. Lower panels show the crystal structures, with the H_C domain in grey, the H_N domain in lavender and the LC domain in cyan. Adapted from Masuyer et al. (2017).

The structural makeup of BoNTs /B and /E largely follows that of BoNT/A, as they are 150 kDa proteins composed of a heavy and light chain, defined functionally into the receptor binding, translocation and catalytic domains (see fig 4.1). They also follow the double receptor mechanism of BoNT entry by binding to gangliosides and synaptic proteins, BoNT/B binding to synaptotagmin I and II, to enter cells and cleave its SNARE target VAMP-2. BoNT/E has the same receptor and SNARE target as BoNT/A, however the site of hydrolysis on the SNAP-25 protein has been shown to result in functional differences in their inhibition of neurotransmitter release (Beske et al., 2017).

BoNT/E cleavage of SNAP-25 results in removal of 17 more residues than BoNT/A cleavage (see fig 4.2). Beske *et al* (2017) showed that this shorter fragment meant cellular neurotransmission could not be rescued with calcium ionophores and voltage gated calcium channel (VGCC) agonists as was possible in BoNT/A intoxicated cells (Beske et al., 2017). This may suggest BoNT/E would induce more persistent neuroparalysis than BoNT/A, however the opposite has been shown in human studies where muscles injected with BoNT/E recovered faster than those injected with BoNT/A (Eleopra et al., 1998). While the BoNT/E induced silencing of efferent neurotransmission has been shown widely in the literature, it remains unclear whether it is able to directly alter sensory signalling, and how this compares to the effect of BoNT/A.

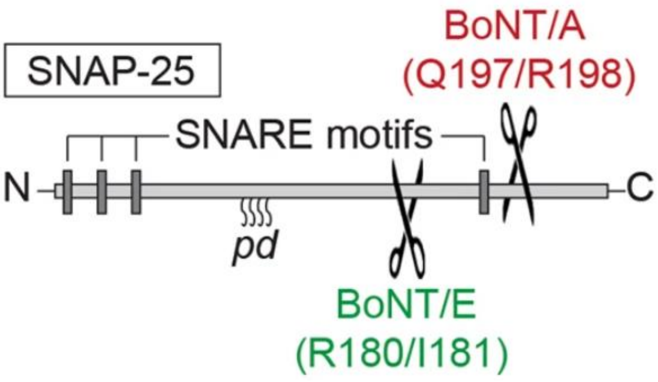


Figure 4.2: Schematic diagram of SNAP-25 and the cleavage sites targeted by BoNTs /A and /E. Note that BoNT/E cleavage results in a shorter fragment than BoNT/A cleavage. Adapted from Beske et al. (2017).

The effects of BoNT/B on afferent neurotransmission has received more investigation than that of BoNT/E, most likely due the availability of the FDA approved formulation Myobloc®. Marino *et al* (2014) conducted an *in vivo* study assessing the antinociceptive potential of BoNT/B following intraplantar injection of capsaicin and formalin, finding significantly reduced flinching and plasma extravasation in treated mice (Marino et al., 2014). A similar study by Huang *et al* (2011) found intrathecal delivery of BoNT/B significantly reduced formalin-induced flinching as well as tactile allodynia associated with spinal nerve ligation (Huang et al., 2011). Both studies found reduced expression of VAMP-2 in the DRG and spinal cord respectively, suggesting intracellular cleavage by BoNT/B. It is unclear whether BoNT/B is able to directly modulate afferent excitability, or if the reduced sensory transmission is due to attenuated release of neurotransmission.

The aim of this study was to assess whether BoNTs /B and /E were able to directly modulate bladder mechanosensation and compare the results to those of BoNT/A treated preparations. This would allow us to better understand the sensory effects of BoNTs and potentially provide evidence regarding the suitability of BoNTs /B and /E for further clinical development.

MATERIALS AND METHODS

Ex vivo extracellular electrophysiology recordings from the mouse bladder

The ex vivo extracellular electrophysiology method is described in detail in chapter 2. Briefly, the urethral catheter was connected to a syringe pump that was programmed to fill the bladder at a rate of 150µL/min. Once 50 mmHg was reached, the tap connected to the dome catheter was opened to allow bladder emptying. A nerve bundle containing pelvic and hypogastric nerves was inserted into a glass electrode to facilitate capture of afferent nerve responses to bladder stimulation. All ramp distensions were performed 10 minutes apart, and each preparation was allowed to stabilise for at least 30 minutes before recording started.

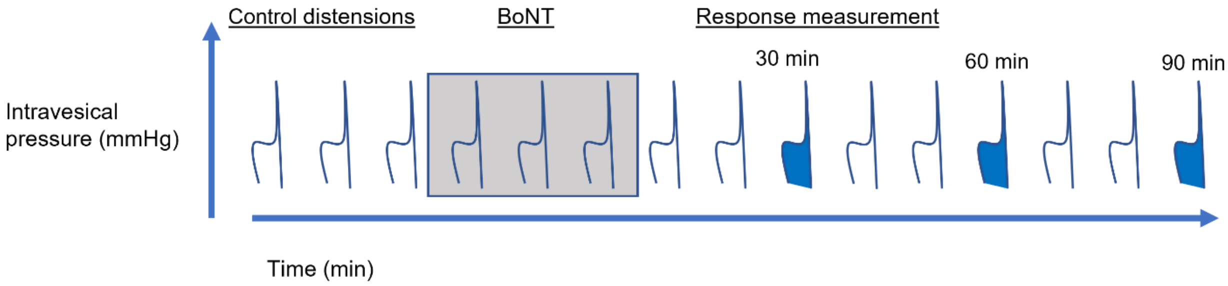


Figure 4.3: A schematic diagram of the distension protocol followed. Distensions were defined as rises intravesical pressure to 50 mmHg induced by bladder filling through the syringe pump, which dropped sharply once the tap was opened. All distensions were 10 minutes apart, starting with intravesical phosphate buffered saline (PBS) during the three control distensions, followed by BoNT in vehicle PBS (shaded), then back to PBS in the response distensions. The filled blue distensions correspond to the 30, 60 and 90 minute time points post-BoNT used in the analysis.

Distensions were performed until the distension-induced nerve responses were reproducible, the protocol followed is shown above in figure 4.3. Following three reproducible 50 mmHg distensions using PBS, BoNT/B or BoNT/E were applied intravesically using a syringe pump, the bladder was distended three times at a speed of 150 µL/min to 50 mmHg. After which, distensions were continued with PBS for nine distensions 10 minutes apart.

Preparation of BoNTs

BoNT/B and BoNT/E were purchased from List Biologicals. BoNTs were kept frozen below -20C, on each day before use vials were defrosted and diluted to the target concentration in phosphate buffered saline (PBS) in a Class 2 Microbiological Safety Cabinet following necessary safety protocols.

Preliminary experiments revealed the Dysport concentration to provide robust, reproducible responses to be 100U/ml. The total toxin amount was calculated to be 3.6pM, so to facilitate accurate comparison, all other BoNTs were applied at 3.6 pM.

RESULTS

4.1 The effect of BoNT/B on distension induced afferent firing

Botulinum neurotoxin serotype B (BoNT/B) was instilled intravesically to assess its effect on distension induced afferent firing. As shown in representative figure 4.4, BoNT/B reduced afferent firing in response to distension over time. This was found to be significant in the analysis ($p < 0.0001$; *n* = 6; figure 4.5A). BoNT/B appeared to significantly increase the pressure-volume relationship of the bladder, which is taken as a measure of bladder compliance ($p<0.0001$; figure 4.5B).

To further understand whether BoNT/B acted differentially on low and high threshold nerve population, analyses of peak firing at 15 and 50 mmHg were conducted. There was no difference difference between the inhibition imposed on both low threshold and high threshold afferents, nor was the inhibition time dependent (figure 4.6)

When compared to the inhibitory effect induced by BoNT/A, while BoNT/B appeared to attenuate distension induced afferent firing to a greater degree ($p = 0.049$; figure 4.7)

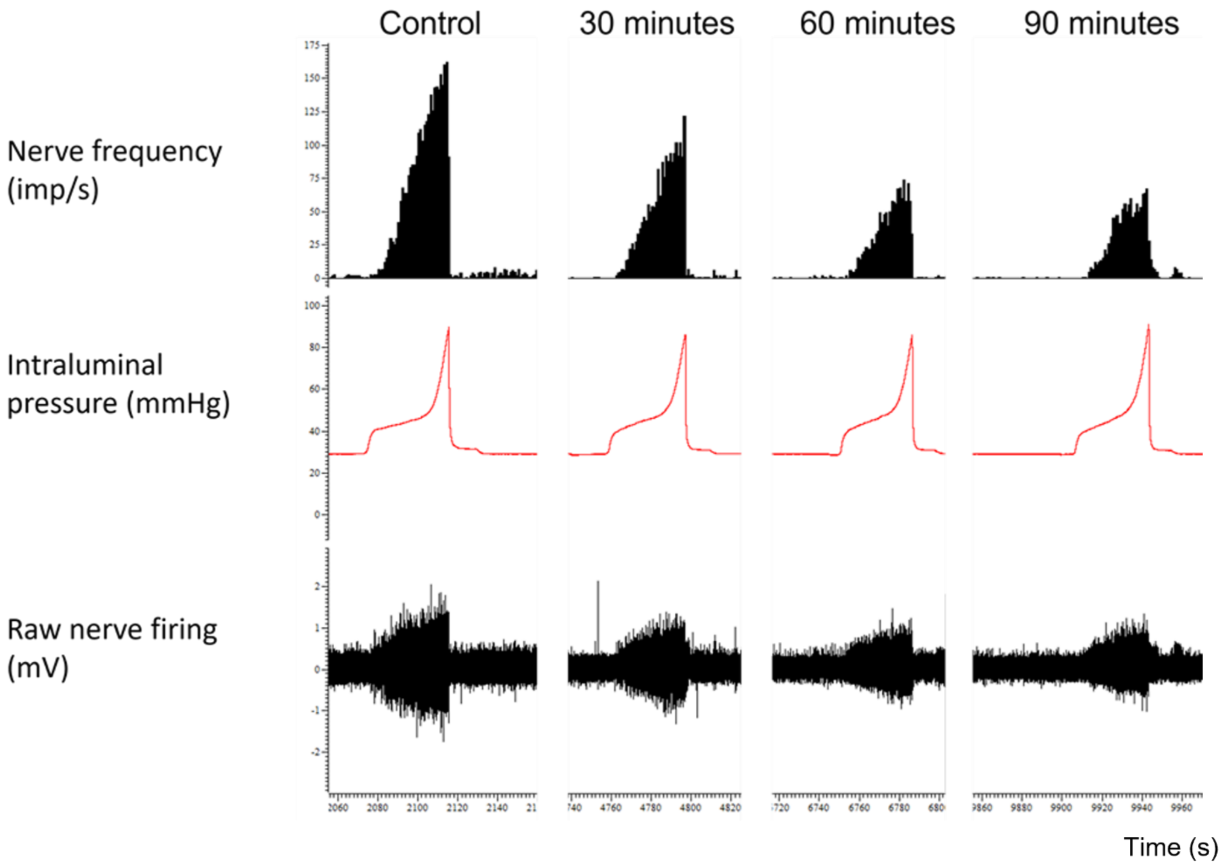


Figure 4.4: The effect of BoNT/B on distension induced firing, an example trace showing the response of afferent nerves to distension prior to (control) and 30, 60 and 90 minutes after application of BoNT/B.

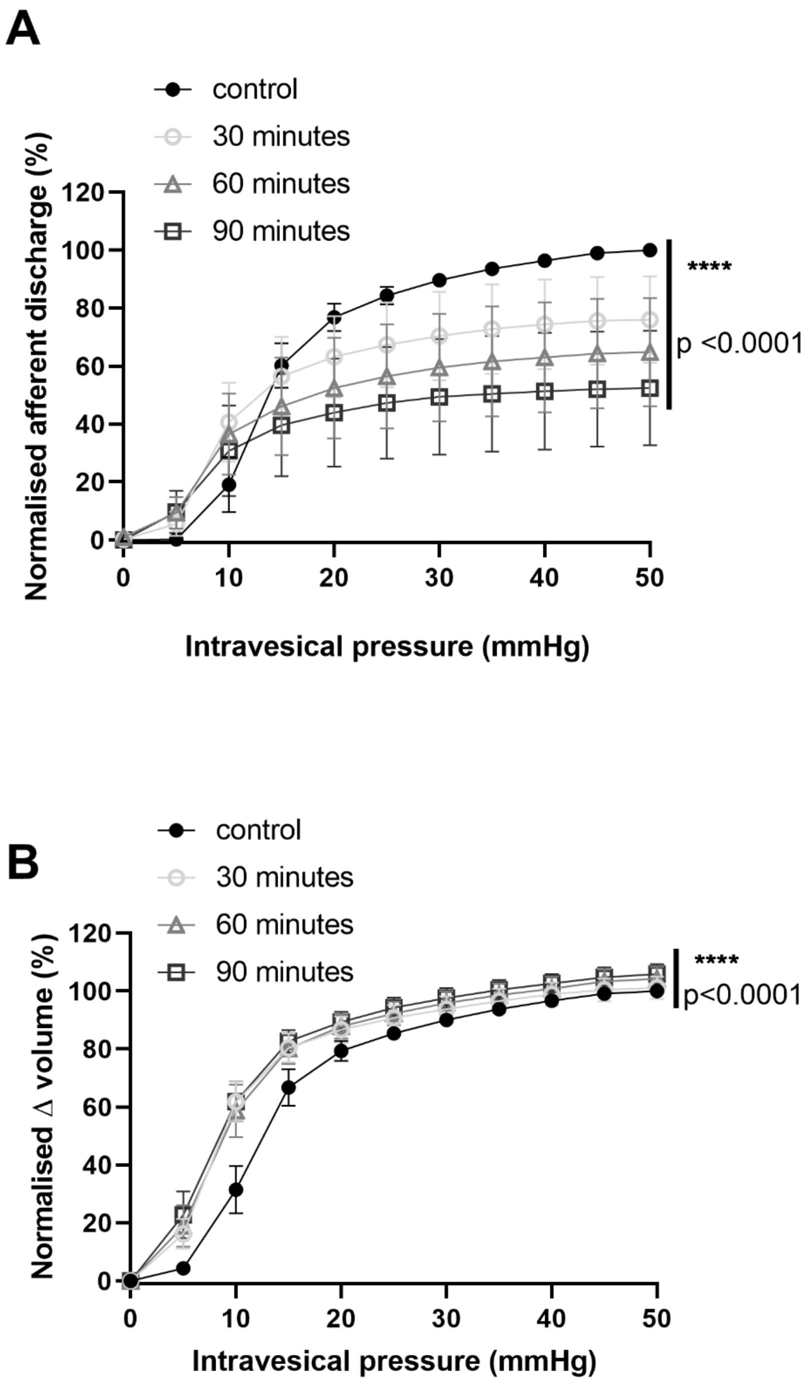


Figure 4.5: The effect of BoNT/B on bladder mechanosensitivity. **A)** Afferent responses to distension were reduced in a time dependent manner following BoNT/B treatment ($p<0.0001$; n = 6; two-way ANOVA). **B)** the pressure-volume relationship was significantly higher following BoNT/B treatment ($p <0.0001$; two-way ANOVA)

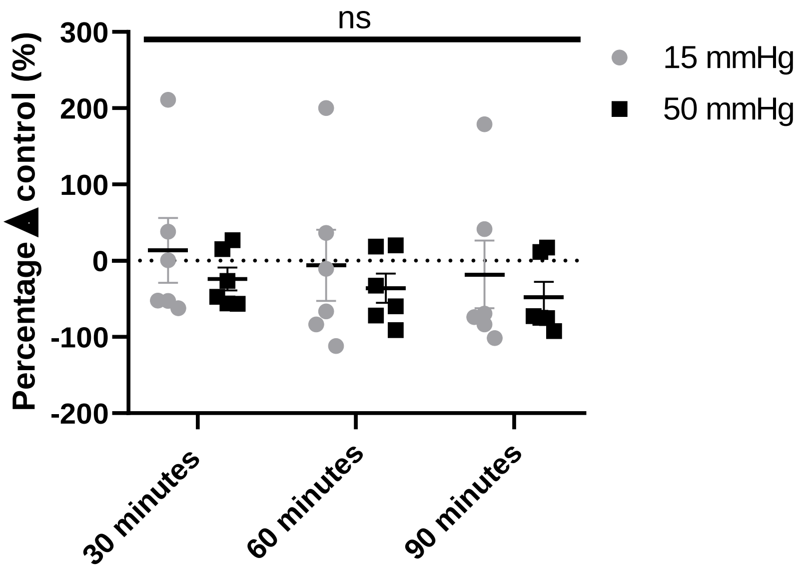


Figure 4.6: Peak firing did not show significant time dependent reduction at 15 mmHg and 50 mmHg (p = 0.3190; *n* = 6; two-way ANOVA).

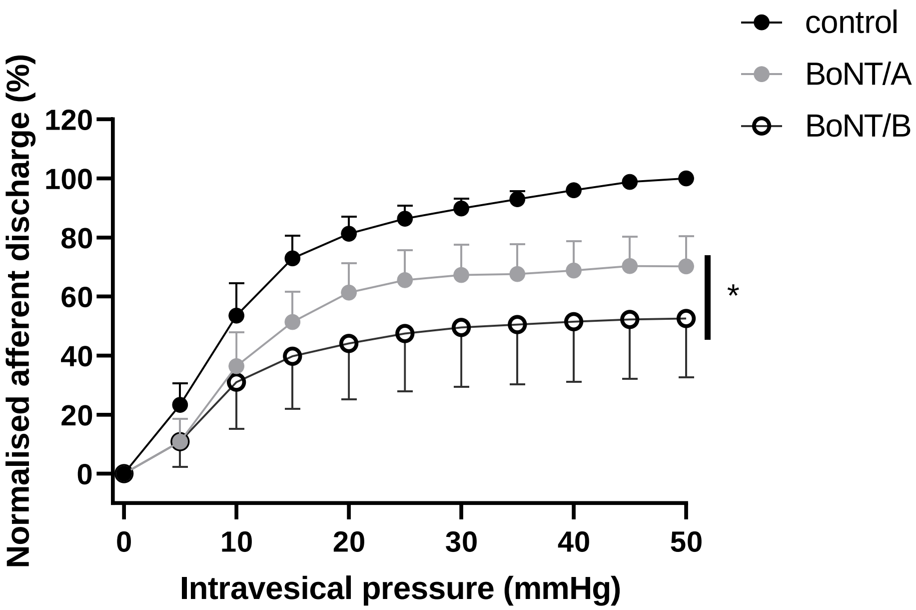


Figure 4.7: Comparison of the inhibitory effect on mechanosensation induced by BoNTs /A and /B. A) BoNT/B inhibited distension induced firing more potently than BoNT/A after 90 minutes (p = 0.049; BoNT/A *n* = 5; BoNT/B *n* = 6; two-way ANOVA).

4.2 The effect of BoNT/E on distension induced afferent firing

Next, the effect of BoNT/E on bladder mechanosensitivity was investigated. Figure 4.8 below shows a representative recording of distension induced responses following treatment with BoNT/E. Distension induced afferent firing was significantly reduced by BoNT/E ($p<0.0001$; figure 4.9A; *n* = 5). Bladder compliance was significantly increased following BoNT/E treatment, as significantly higher volume was needed to reach 50 mmHg at the end of the experiment as compared to the control distension at the beginning ($p<0.0001$; figure 4.9B).

Intravesical BoNT/E did not induce time dependent loss in afferent activity, in both low threshold (15 mmHg) and high threshold (50 mmHg) responding nerve fibres (figures 4.10).

The effect of BoNT/E on bladder mechanosensitivity was compared to that of BoNT/A, revealing more potent inhibition ($p = 0.0084$; figure 4.11).

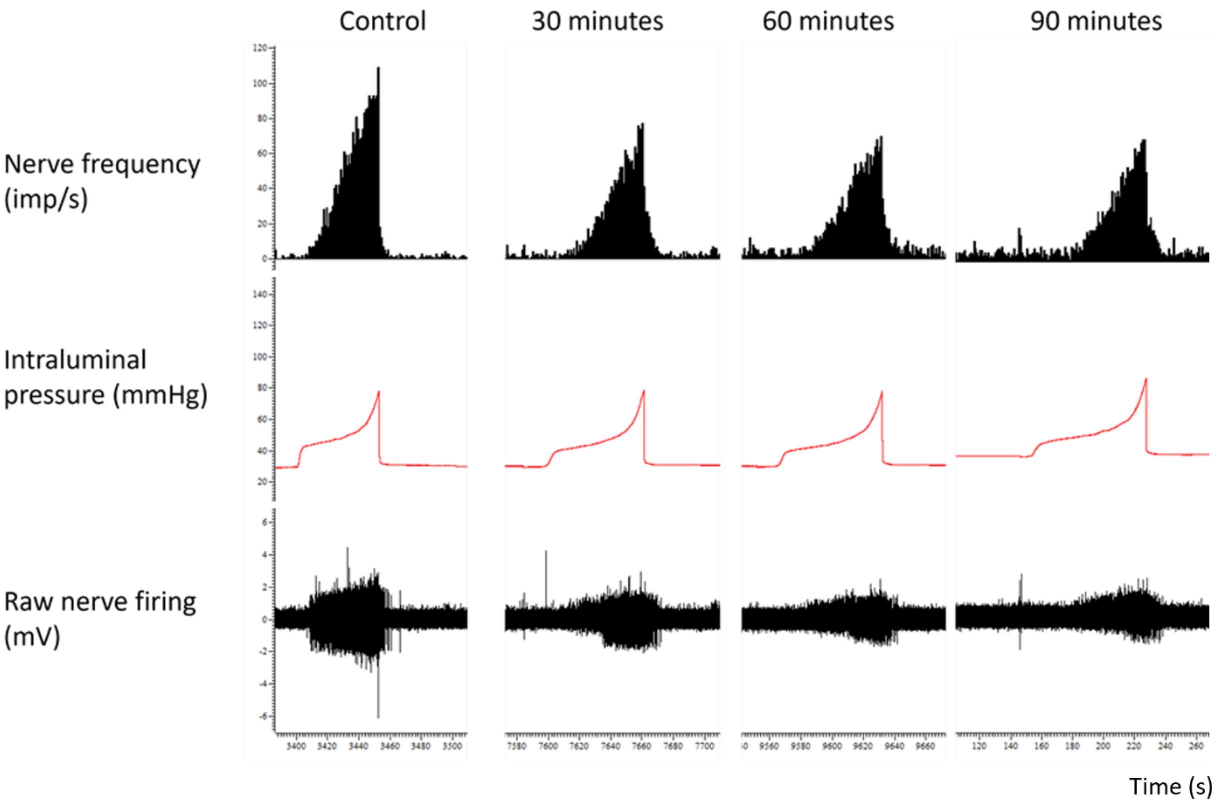


Figure 4.8: a representative trace showing the effect of BoNT/E on distension induced firing over time

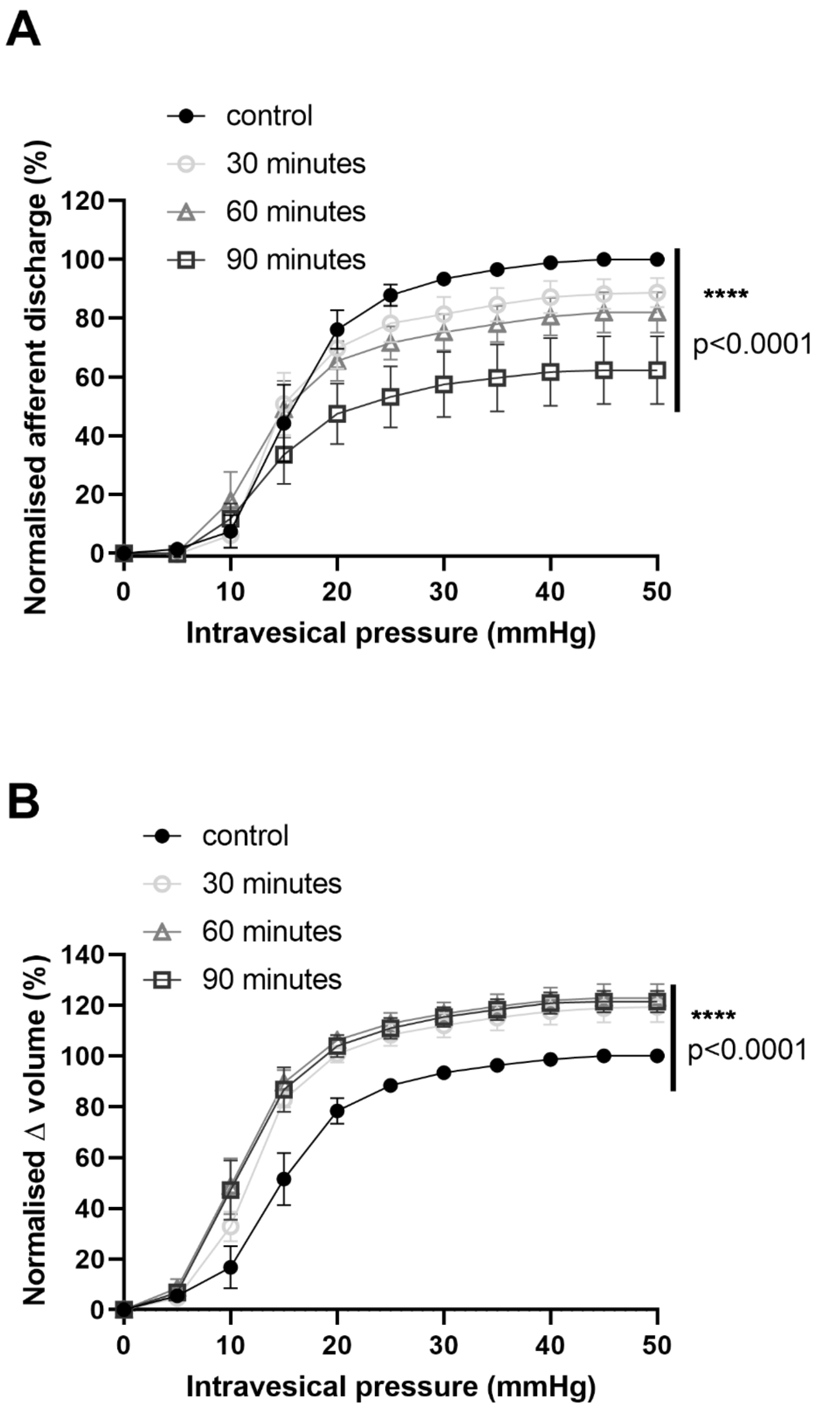


Figure 4.9: The effect of BoNT/E on bladder mechanosensitivity. **A)** Afferent responses to distension were significantly reduced in a time dependent manner following BoNT/E treatment (p<0.0001; n = 5; two-way

ANOVA). **B)** the pressure-volume relationship was significantly higher following BoNT/E treatment (p <0.0001; two-way ANOVA)

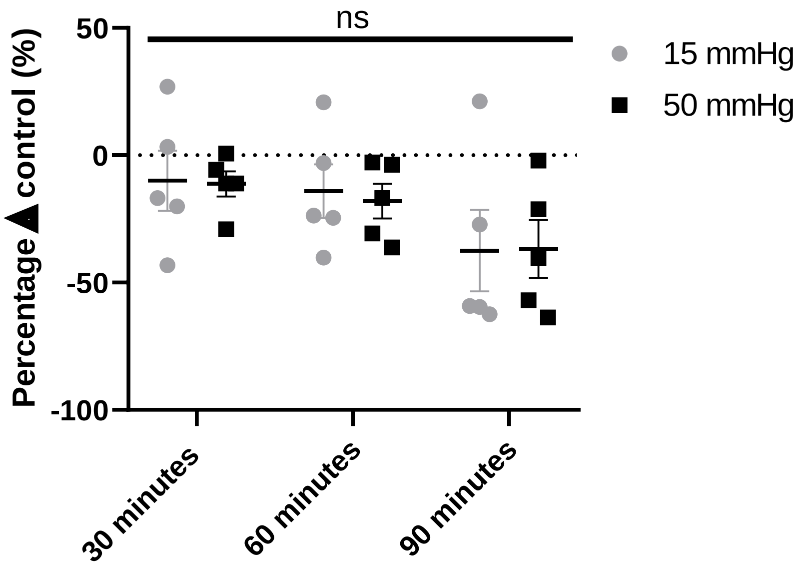


Figure 4.10: Peak firing at 15 mmHg and 50 mmHg did not show a time dependent reduction (p = 0.8263; *n* = 5; two-way ANOVA).

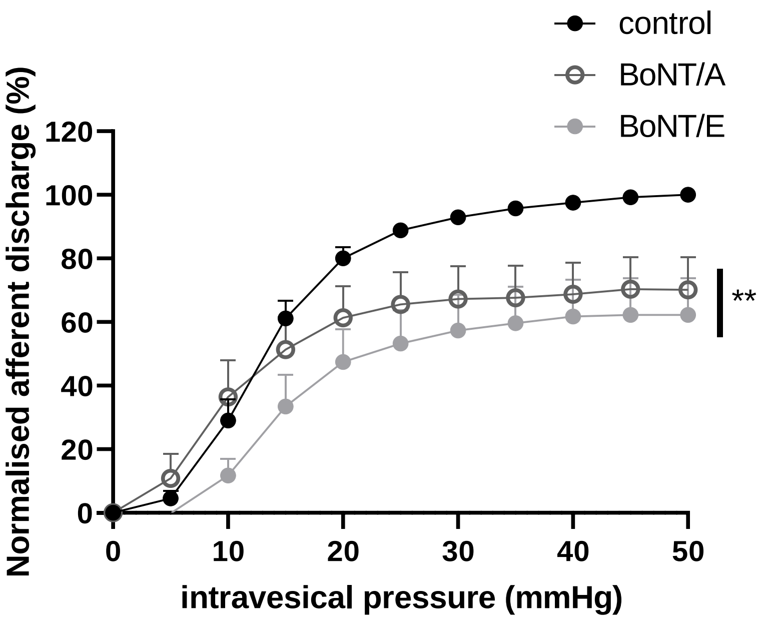


Figure 4.11: Comparison of the inhibitory effects on mechanosensitivity induced by BoNTs /A and /E. BoNT/E inhibited distension induced firing more potently than BoNT/E after 90 minutes (p = 0.0084; BoNT/A *n* = 5; BoNT/E *n* = 5; two-way ANOVA)

DISCUSSION

The main findings reported in this chapter include

- BoNTs /B and /E significantly attenuated distension induced afferent firing, more potently than BoNT/A.

The results of this investigation have revealed direct effects of BoNTs /B and /E on bladder mechanosensation for the first time. This provides further evidence regarding the activity of BoNTs in the modulation of bladder sensation, confirming bladder mechanosensation is susceptible to multiple serotypes of BoNT.

BoNT/B significantly attenuated bladder mechanosensation

While the effect of BoNT/A in modulating bladder sensory signalling has been widely studied, there has been less attention paid to the other serotypes. The aim of this study was to characterise BoNT/B and understand whether it can directly affect the detection and transduction of sensory signals. This is important not only to provide a potential treatment alternative to BoNT/A, but also to use it as a tool to better understand the mechanisms underlying visceral mechanotransduction, which remain elusive.

In the present study, BoNT/B was found to significantly inhibit distension induced responses, as by 90 minutes following intravesical BoNT/B treatment, 52.3% (+/- 19.8%) of afferent firing remained (figure 4.5A). This data provided evidence that shows BoNT/B can directly inhibit the ability of the bladder to sense mechanical distension. The *ex vivo* electrophysiology assay also provided information regarding the intravesical volume required to reach a specific pressure, which is taken as a measure of bladder compliance. In this study, BoNT/B caused a significant and maintained increase in the pressure-volume relationship (figure 4.5B). It is currently unclear how this can occur due to the *ex vivo* nature of this preparation, which means there is no active efferent input that may be targeted by BoNT/B. This data is difficult to interpret due to the significant increase in bladder compliance seen in control preparations (see methods figure 2.4), which means any additional effects of BoNT/B are not clear. As discussed in the previous chapter, bladder elasticity and accommodation of volume depends on the extracellular matrix proteins and detrusor smooth muscle cells, which may be altered by BoNT application. There is also the potential alteration in the release of a nonadrenergic noncholinergic (NANC) neurotransmitter which may act to modulate detrusor tension to increase bladder compliance. This is explored in further detail in chapter five.

As this data shows BoNT/B is able to directly inhibit afferent responses, it may provide an explanation for its antinociceptive and anti-allodynic effects seen in the literature (Huang et al., 2009; Huang et al., 2011; Marino et al., 2014; Park et al., 2015; Sikandar et al., 2016) Previous studies have investigated the action of BoNT/B on the function of primary afferent nerves, Meng *et al* (2007) found BoNT/B significantly decreased K^+ evoked release of neuropeptides from mouse trigeminal neurons (Meng et al., 2007). These findings suggest BoNT/B mediated cleavage of VAMPs inhibit the release of neuropeptides such as CGRP which in turn halts the progression of sensory signals. This may stop the development of peripheral sensitization and hyperalgesia.

However, a question that remains is whether cleavage of VAMPs may directly inhibit afferent excitation, due to the findings in the present study of BoNT/B dependent reduction of mechanosensory signalling. Interestingly, BoNT/B appeared to induce significantly more potent attenuation of afferent responses than BoNT/A (figure 4.7). The difference in responses found after treatment with BoNTs /A and /B in the bladder has been reported previously in a different experimental model. Maignel-Ludop et al (2017) conducted a study on BoNT/A and /B on mouse bladder strip contractility, finding BoNT/B inhibited contractility significantly more potently than BoNT/A (Maignel-Ludop et al., 2017). These findings cannot be directly compared due to the difference in experimental setup, as in the present study there is no active efferent input, however, it may be possible that BoNT/B is more active in the mouse bladder due to increased expression of its SNARE target within the bladder wall. The expression of VAMP mRNA has been shown in the porcine urothelium (Bahadory et al., 2014), however western blots performed on mouse urothelial cells showed no expression (Wankel et al., 2016), while there does not appear to be data available on the expression of VAMP-2 in other cell types. These differences may be species dependent, or it may be due to differences in antibodies used. Currently, it is difficult to make conclusions using the data outlined in this chapter as to the mechanism by which BoNT/B inhibits bladder afferent signalling, however, this may be possible through further investigations in this field.

In this thesis, there is evidence showing BoNT/A mediated inhibition may be SNARE independent, which may enable speculation on whether other BoNTs can inhibit sensory signalling in a SNARE independent manner. In chapter five, a catalytically inactive recombinant version of BoNT/A was tested in the *ex vivo* bladder electrophysiology assay, to investigate any SNAP-25 independent activity. In a similar fashion, a similar catalytically inactive construct of BoNT/B may provide answers as to whether cleavage of VAMP-2 is involved in the afferent inhibition seen in this chapter.

Despite the lack of basic science studies investigating the function of BoNT/B in the bladder, clinical studies have been conducted to assess its utility in urology. Dykstra *et al* (2003) conducted a pilot study investigating the effects of BoNT/B on OAB symptoms, reporting significant reductions in urgency, frequency and incontinence episodes (Dykstra et al., 2003). These data are supported by findings by Ghei *et al* (2005) and Hirst *et al* (2007), who reported significant changes in voided volume, frequency and incontinence episodes following intravesical injection of BoNT/B in humans (Ghei et al., 2005; Hirst et al., 2007). All three studies reported short duration of action, the shortest being Hirst *et al* (2007) who found that patients reported urgency symptoms returning at the 10-week assessment (Hirst et al., 2007). These studies support the findings in the present study that intravesical BoNT/B application causes reduced afferent transmission however, due to the 90-minute duration of the experiments, it is not possible to say whether this inhibition remained long term. There does not seem to be any clinical studies that find BoNT/B to have higher efficacy than BoNT/A as found in the present chapter. This may question the translatability of the findings, however, it can also be explained by the fact that a point mutation in the human synaptotagmins I and II results in reduced affinity of BoNT/B for human cells (Peng et al., 2012). Due to the reduced uptake of BoNT/B in human cells, higher dosages are necessary to provide similar therapeutic effects as BoNT/A, for example studies of patients with cervical dystonia have shown bioequivalent units of BoNT/B to BoNT/A to be between 40:1 and 70:1 (Pappert & Germanson, 2007; Tintner et al., 2005; Truong et al., 1991). These higher doses have been associated with increased risk of immunogenicity and stimulation of neutralizing antibodies (Jankovic et al., 2006), however, long-term studies have shown presence of antibodies does not appear to correlate with a loss of therapeutic effect (Chinnapongse et al., 2012).

These clinical findings support the view that there may be some therapeutic value in the use of BoNT/B. The reduction in urgency induced by BoNT/B injection suggests there may be direct sensory effects, which has not received detailed investigation. If BoNT/B is able to directly inhibit sensory afferents, it may be of clinical relevance as an alternative to BoNT/A.

BoNT/E significantly attenuated bladder mechanosensation

This is the first study showing the effects of BoNT/E on bladder afferent nerve signalling. In this study, it was used to better understand the function of SNAP-25 in the detection and propogation of sensory signals, and whether this is affected by cleavage at a different site on the protein. As previously discussed, BoNT/E leaves a shorter fragment of SNAP-25 within the cell, and in the motor neuron, this has been shown to cause a more transient neuroparalysis than BoNT/A. The aim of the present study was to characterise BoNT/E in the *ex vivo* preparation and show whether the difference in cleavage site has functional differences in bladder sensation.

In this chapter, significant inhibition of distension induced afferent firing in response to intravesical instillation of BoNT/E was found (figure 4.9A). This data confirms BoNT/E can directly inhibit bladder mechanosensation, which adds to the number of BoNTs that have been shown to modulate afferent responses. Similarly to BoNT/B, BoNT/E also induced a significant increase in bladder compliance, however, as previously discussed, due to the significant increase observed in control preparations it is not clear to what extent BoNT/E was able to modulate compliance. This can be further investigated in future studies.

When compared to the BoNT/A induced inhibition, BoNT/E was significantly more effective at reducing distension induced afferent firing (figure 4.11). This data suggests that cleavage of SNAP-25 by BoNT/E may cause more disruption to afferent responses than that of BoNT/A. This hypothesis is supported by Meng *et al* (2009) who showed that 17 amino acid residues between the SNAP-25 cleavage sites of BoNTs /A and /E are responsible for the binding to syntaxin and formation of the SNARE complex (Meng et al., 2009). In the present study, the presence of cleaved SNAP-25 in the BoNT/E treated preparations was not investigated, as the cSNAP-25 antibody used in chapter three may not have been able to recognise the shorter fragment left by BoNT/E cleavage. This makes it difficult to confidently attribute the difference in response to SNAP-25 cleavage, which is further complicated by the findings in the subsequent chapter that BoNT/A is capable of modulating afferent neurotransmission independently of SNAP-25 cleavage.

Although BoNT/E appears to be more active in inducing afferent inhibition than BoNT/A, it has been shown to be less effective at entering cells. While BoNT/E also binds to SV2 to enter cholinergic neurons, it preferentially targets glycosylated SV2 A/B which is less expressed in neurons than the SV2C targeted by BoNT/A (Meng et al., 2009). It remains unclear which versions of SV2 are expressed in urothelial cells, afferent terminals, interstitial cells and smooth muscle, it may be the case that the SV2 targeted by BoNT/E is more highly expressed than that targeted by BoNT/A, explaining the higher efficacy. This requires further study to improve our understanding.

To increase the number of cell types that can be targeted by the BoNT/E light chain (LC/E), Meng et al (2009) designed a chimera of BoNTs /A and /E containing the receptor binding region of /A and the catalytic region of /E. They found while both BoNT/A and BoNT/E were ineffective at blocking the capsaicin induced CGRP release from trigeminal neurons individually, the /EA chimera was capable of blocking CGRP release (Meng et al., 2009). Multiple groups have conducted similar studies retargeting the LC/E into cells to take advantage of its increased effectiveness at cleaving SNAP-25. Recently, Antoniazzi *et al* (2022) produced a chimera of the LC/E attached to the whole BoNT/A protein that attenuated capsaicin induced nocifensive behaviour once injected into the whisker pad of rats (Antoniazzi et al., 2022.). Using herpes simplex virus vectors, Joussain *et al* (2019) injected the light chains of BoNTs /A to /F into primary DRG neurons, finding they all inhibited the evoked release of CGRP (Joussain et al., 2019). These studies exemplify the ways BoNTs have been modified in recent years to further take advantage of the potent neuroparalytic activity to improve their antinociceptive function. Investigating the function of these chimeras on the afferent signalling in the bladder may provide evidence to inform further treatments for overactive bladder, as currently the only BoNT approved by regulatory agencies across the world is BoNT/A. Improving the entry of BoNTs into cells using new vehicles or mechanisms could bypass the need for invasive techniques such as injection.

CONCLUSION

This chapter presented data characterizing BoNT serotypes /B and /E in an *ex vivo* bladder preparation, showing significant direct effects on bladder sensation. These findings were novel and provide new perspective on the utility of BoNTs in the bladder, proposing that there may be therapeutic potential to be found outside of BoNT/A.

CHAPTER 5 – THE EFFECTS OF CATALYTICALLY INACTIVE BONT/A ON BLADDER MECHANOSENSITIVITY

INTRODUCTION

The discovery of the therapeutic potential of BoNT/A decades ago has led to its use for the treatment of disorders including cervical dystonia and overactive bladder. Further development of the molecule has been hampered by the safety issues, it remains the most potent natural neurotoxin in the world with a lethal dose in nanograms per kilogram (Gill, 1982). To better understand molecular functions of BoNTs, as well as investigate potential uses in drug delivery, non-toxic BoNTs without the ability to cleave their SNARE targets have been developed.

BoNTs owe their potent neuroparalytic activity to the zinc metalloprotease function of the light chain domain (Schiavo et al., 1992). The importance of Zn^{2+} in facilitating cleavage of SNARE proteins was discovered after investigators found that removal of Zn^{2+} by chelators inhibited the biological activity of the BoNT/A molecule (Fu et al., 1998; Schiavo et al., 1992; Simpson et al., 2001). All seven BoNT light chains (/A to /G), as well as that of tetanus toxin, require Zn^{2+} to exert their proteolytic activity (Lebeda et al., 2010). Zn^{2+} metalloproteases have a common motif, the HEXXH sequence shown below in figure 5.1, where a catalytic Zn^{2+} ion interacts with two histidine and one glutamine residue, as well as a water molecule (Schiavo et al., 1992).

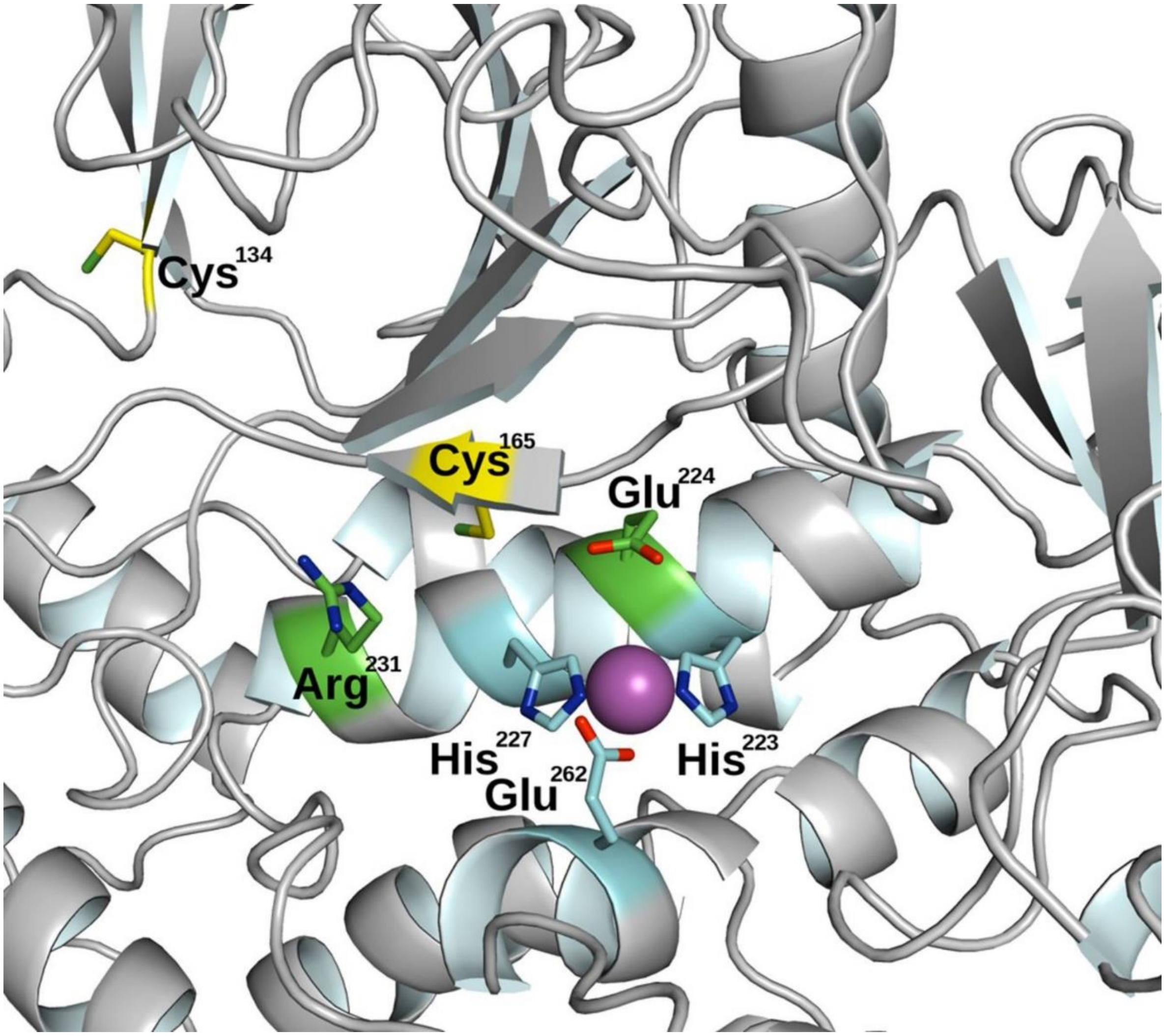


Figure 5.1: Composition of the catalytic domain of BoNT/A**:** the catalytic Zn^{2+} ion (represented by a purple sphere) is chelated by His223, His227 and Glu262 residues, shown above in cyan. The Arg231 and Glu224 residues that have also been reported to play a part in catalytic activity are shown in green, and cysteine residues in yellow. Adapted from Stura et al (2012).

Groups interested in developing non-toxic BoNTs have induced mutations in the HEXXH motif of the light chain, where any of the residues important for SNARE cleavage are substituted for a different amino acid to create a non-functional catalytic domain. Rigoni *et al* (2001) took a site directed mutagenesis approach to create targeted changes in the light chain to find the amino acid residues essential for SNARE proteolysis. They found that replacing Glu^{262} with an alanine residue significantly decreased catalytic activity of BoNT/A (Rigoni et al., 2001). Li & Singh (2000) substituted the Glu^{224} residue with glutamine and discovered total loss of endopeptidase activity, while aspartic acid led to only 1.4% of enzymatic activity remaining (Li & Singh, 2000). Krukeja *et al* (2007) found replacement of Glu^{262} with glutamine led to structural alterations, as zinc binding and substrate binding were impaired, as well as loss of catalytic activity (Kukreja et al., 2007).

The aim of this study was to use a form of recombinant inactive BoNT/A, termed BoNT/A (0), to characterise the SNAP-25 cleavage independent actions of BoNT/A on bladder function. To achieve this, the effects of BoNT/A (0) and a light chain only construct LC/A (0) on distension induced afferent firing were investigated. Also, whether BoNT/A (0) pretreatment had any effect on capsaicin induced afferent firing and contraction was investigated, as a measure of its effects on bladder chemosensation.

MATERIALS AND METHODS

Ex vivo extracellular electrophysiology recordings from the mouse bladder

The ex vivo extracellular electrophysiology method is described in detail in chapter 2. Briefly, the urethral catheter was connected to a syringe pump that was programmed to fill the bladder at a rate of 150µL/min. Once 50 mmHg was reached, the tap connected to the dome catheter was opened to allow bladder emptying. A nerve bundle containing pelvic and hypogastric nerves was inserted into a glass electrode to facilitate capture of afferent nerve responses to bladder stimulation. All ramp distensions were performed 10 minutes apart, and each preparation was allowed to stabilise for at least 30 minutes before recording started.

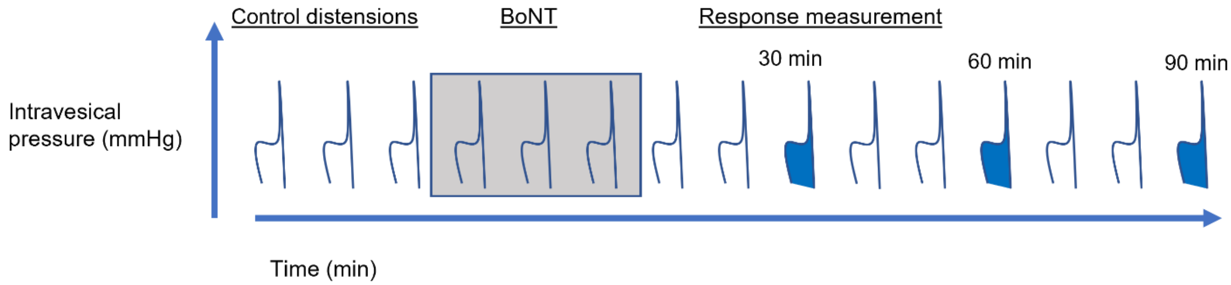


Figure 5.2: A schematic diagram of the distension protocol followed. Distensions were defined as rises intravesical pressure to 50 mmHg induced by bladder filling through the syringe pump, which dropped sharply once the tap was opened. All distensions were 10 minutes apart, starting with intravesical phosphate buffered saline (PBS) during the three control distensions, followed by BoNT in vehicle PBS (shaded), then back to PBS in the response distensions. The filled blue distensions correspond to the 30, 60 and 90 minute time points post-BoNT used in the analysis.

Distensions were performed until the distension-induced nerve responses were reproducible, the protocol followed is shown above in figure 5.2. Following three reproducible 50 mmHg distensions using PBS, full-length BoNT/A (0) or its associated fragments were applied intravesically using a syringe pump, the bladder was distended three times at a speed of 150 µL/min to 50 mmHg. After which, distensions were continued with PBS for nine distensions 10 minutes apart.

Preparation of BoNT/A (0) and domain fragments

The production of recombinant BoNTs are described in detail in chapter two. Briefly, recombinant BoNT/A (0) and LC/A (0) were produced in-house at Ipsen, the gene sequences were cloned into vectors, expressed in *E. coli* then purified and activated. Mutations in the light chain that rendered them inactive in the HEXXH region of the light chain included substitution of glutamic acid to glutamine (E224Q), and histidine to tyrosine (H227Y). BoNTs were kept frozen at 80°C, on each day before use vials were defrosted and diluted to the target concentration in phosphate buffered saline (PBS) in a Class 2 Microbiological Safety Cabinet following necessary safety protocols.

Preliminary experiments revealed the Dysport concentration to provide robust, reproducible responses to be 100U/ml. The total toxin amount was calculated to be 3.6pM, so to facilitate accurate comparison, all other BoNTs were applied at 3.6 pM.as well as higher and lower concentrations.

Validation of the catalytic inactivity of BoNT/A (0)

The effect of BoNT/A (0) on the function of the neuromuscular junction was assessed in the mouse phrenic nerve hemidiaphragm assay and compared to that of BoNT/A. This assay is a well-characterised method to study the neuromuscular junction (NMJ) and the impact of neurotoxins on its function, by recording evoked muscle contractility directly. Electrical stimulation of the phrenic nerve leads to the SNAP-25 dependent release of ACh at the NMJ which acts on the diaphragm muscle to induce contraction. Due to the purported lack of SNAP-25 cleavage activity of BoNT/A (0), it was important to prove that this translated to the loss of neuroparalytic activity. These experiments were performed by Dr Kevin Retailleau (IPSEN employee) and are included in this thesis to contextualise BoNT/A (0) and provide evidence on its lack of paralytic activity. All hemidiaphragm assay data was collected, analysed and reported by Dr. Retailleau.

Mouse phrenic nerve hemidiaphragm assay

Methods

C57BL/6J mice (5 weeks old) were used in this assay. The left hemidiaphragm and phrenic nerve was dissected and transferred into an organ bath (Emka Technologies, Paris, France). The muscle was connected to a transducer to facilitate recording of muscle contraction. Electrical stimulation was applied to the phrenic nerve (frequency = 1 Hz; width = 20µs; 10V). Following stabilisation and control steps, the sample was incubated with 100 pM recombinant BoNT/A (0) (rBoNT/A (0)), 100 pM recombinant BoNT/A1 (rBoNT/A1) or buffer control (0.1% BSA in PBS) for three hours. Potency was expressed as the time taken to reach half paralysis (t50) using non-linear regression analysis (GraphPad Prism v8.3). All data were expressed as mean +/- SEM, N corresponded to number of animals.

Results

The results are expressed as percentage of the force developed by the hemidiaphragm before the addition of BoNTs or buffer. Over the course of the experiment, contraction amplitude was inhibited completely by rBoNT/A, with a t50 of 24.1 +/- 0.1 minutes. There was no difference between the rBoNT/A (0) molecule and the buffer control, as they retained 84.76 +/- 1.75% and 83.29 +/- 1.26% of preincubation contractility respectively (figure 5.3).

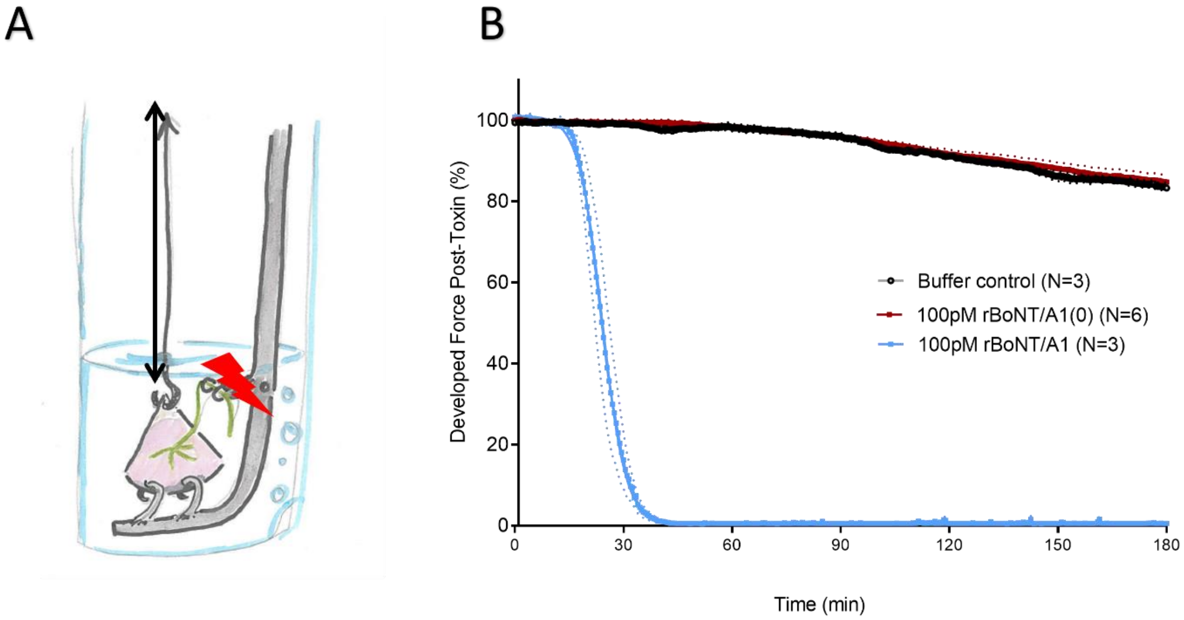


Figure 5.3 The mouse hemidiaphragm assay was used to characterize the neuroparalytic effects of rBoNT/A and rBoNT/A (0). A) a cartoon showing the experimental setup of the hemidiaphragm assay. B) the effect of rBoNT/A1 (*n* = 3) and rBoNT/A (0) (*n* = 6) on hemidiaphragm contractility. Preincubation developed force was inhibited 99.45% (+/- 0.06%) by rBoNT/A1. After three hours of incubation, there was no significant difference between rBoNT/A (0) and the buffer control (*n* = 3).

Conclusion

Based on this *ex vivo* data, 100 pM rBoNT/A (0) had no effect at the neuromuscular junction.

RESULTS

5.1 Effect of catalytically inactive BoNT/A (BoNT/A (0)) on bladder mechanosensitivity

In the present study, a construct of BoNT/A with a non-functional zinc binding region designated BoNT/A (0) was tested to determine its effect on distension-induced afferent firing. As shown in in figures 5.4 and 5.5A, BoNT/A (0) potently inhibited afferent firing in a time dependent manner (p <0.0001; *n* = 8; two-way ANOVA). 30 minutes post-BoNT/A (0) treatment, peak firing was reduced by 37.75% (+/- 6.4%/) of the control distension, which was reduced further by 72.73% (+/- 5.9%) of control at 90 minutes post-treatment (figure 5.5A). To further understand whether BoNT/A (0) acted differentially on low and high threshold nerve population, analyses of peak firing at 15 and 50 mmHg were conducted. Both low (15 mmHg) and high (50 mmHg) responding nerves were significantly inhibited by BoNT/A (0) in a time dependent manner, however there were no differences between inhibition of nerves responding at 15 and 50 mmHg at any time point (p = 0.4648; figure 5.6). BoNT/A (0) appeared to have no effect on the pressure-volume relationship of the bladder, which is taken as a measure of bladder compliance (p= 0.1652; figure 5.5B).

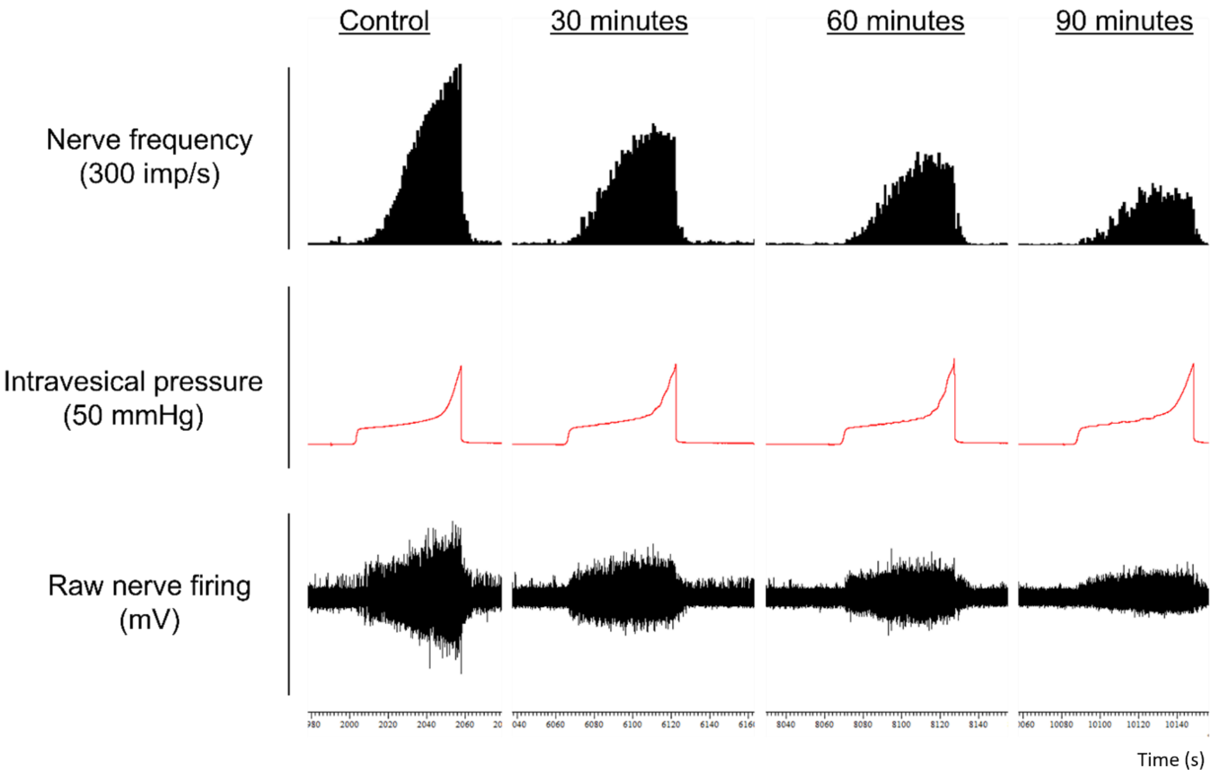
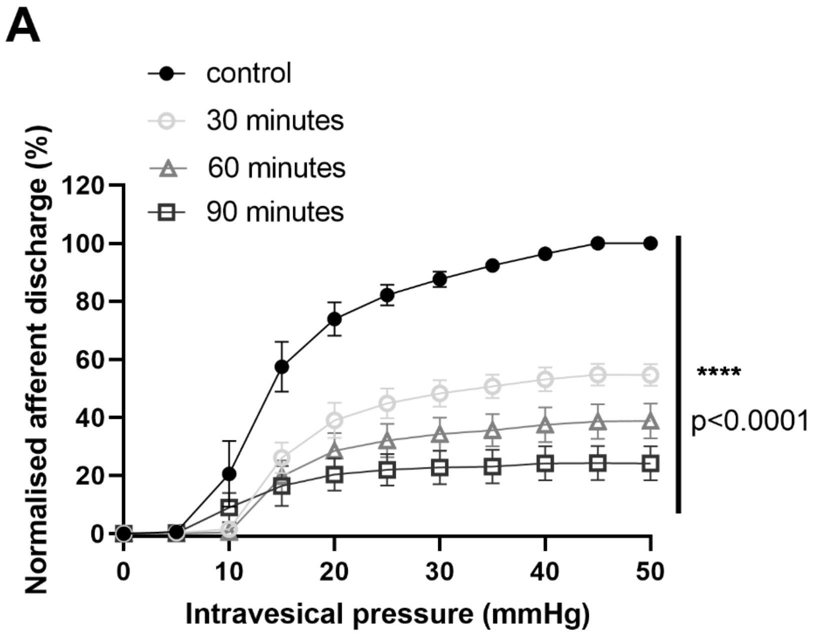


Figure 5.4: The effect of BoNT/A (0) on distension induced firing, an example trace showing the response of afferent nerves to distension prior to (control) and 30, 60 and 90 minutes after application of BoNT/A (0).



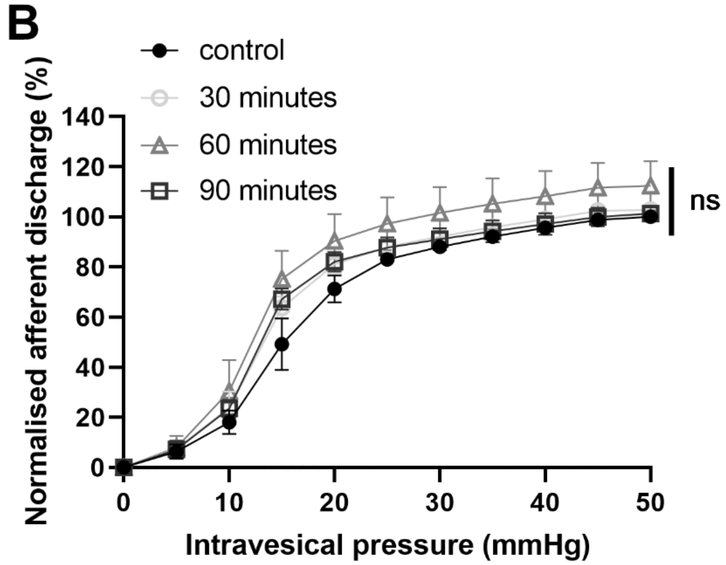


Figure 5.5: BoNT/A (0) significantly inhibits distension induced afferent firing. **A)** Afferent responses to distension were reduced in a time dependent manner following BoNT/A (0) treatment ($p<0.0001$; n = 8;

two-way ANOVA). **B)** The pressure-volume relationship was unchanged following BoNT/A (0) treatment (p = 0.1567; two-way ANOVA)

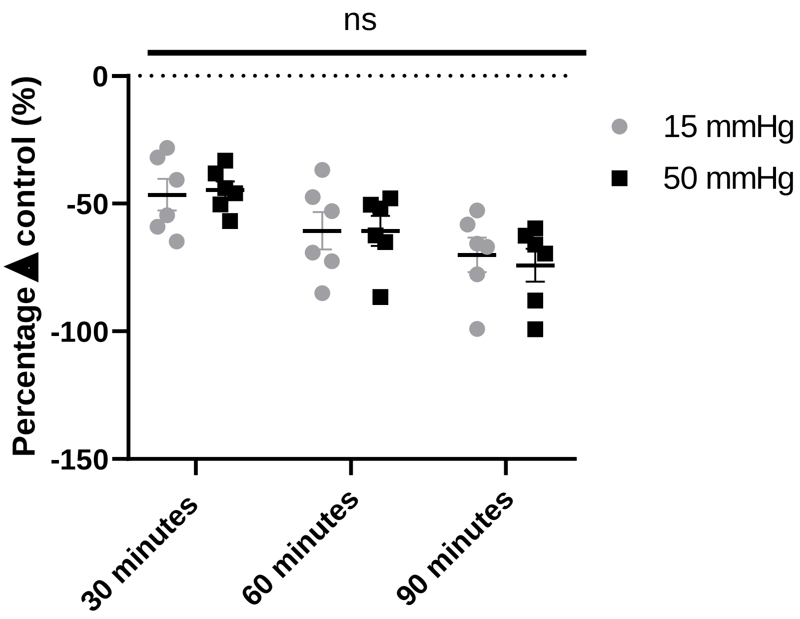


Figure 5.6 BoNT/A (0) did not differentially inhibit low and high threshold firing (p=0.4648; n = 8; mean +/- SEM; two-way ANOVA).

The effect of 1 fM BoNT/A (0) on bladder mechanosensitivity

To further characterise the effects of BoNT/A (0) on distension induced afferent firing, a lower concentration was intravesically instilled. 1 fM BoNT/A (0) significantly decreased afferent firing as by 90 minutes following treatment, only 38.9% (+/- 18.8%) of the nerve response remained (figure 5.7A; p <0.0001; *n* = 4). The afferent inhibition appeared to be time dependent, as responses at 90 minutes were significantly lower than control. To look separately at the effect of 1fM BoNT/A (0) on low threshold and high threshold firing, the peak responses at each respective pressure was compared to that of the control distension at the beginning of the experiment. Peak firing at 15 mmHg and 50 mmHg was not inhibited in a time dependent manner (p = 0.3429; figure 5.8). Bladder compliance appeared to be significantly increased following treatment with 1fM BoNT/A (0) (figure 5.7B; p <0.0001; *n* = 4).

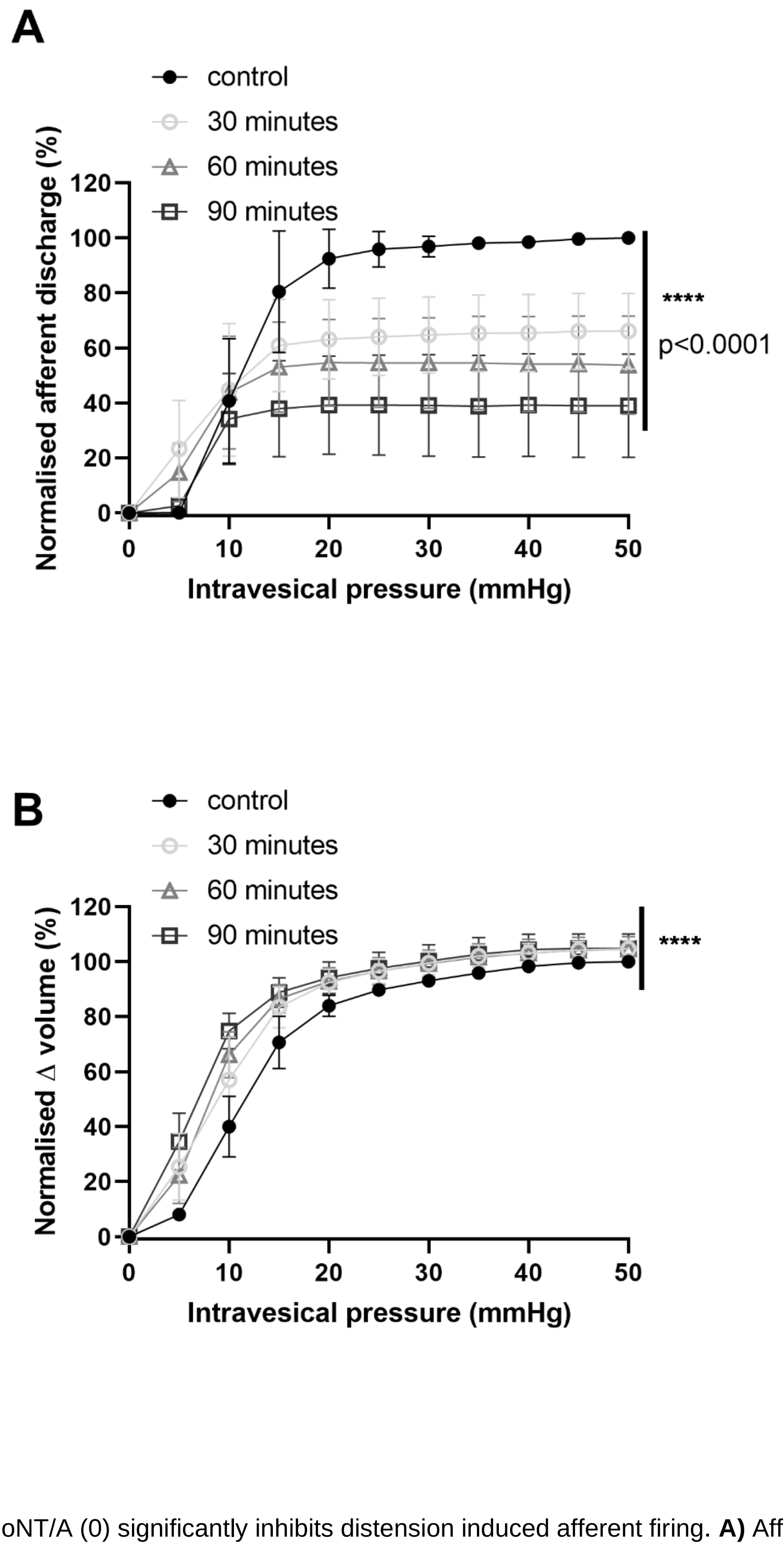


Figure 5.7: 1fM BoNT/A (0) significantly inhibits distension induced afferent firing**. A)** Afferent responses to distension were reduced in a time dependent manner following BoNT/A (0) treatment ($p<0.0001$; n = 4;

two-way ANOVA). **B)** The pressure-volume relationship was significantly increased after intravesical BoNT/A (0) compared to control (p <0.0001; n = 4; two-way ANOVA).

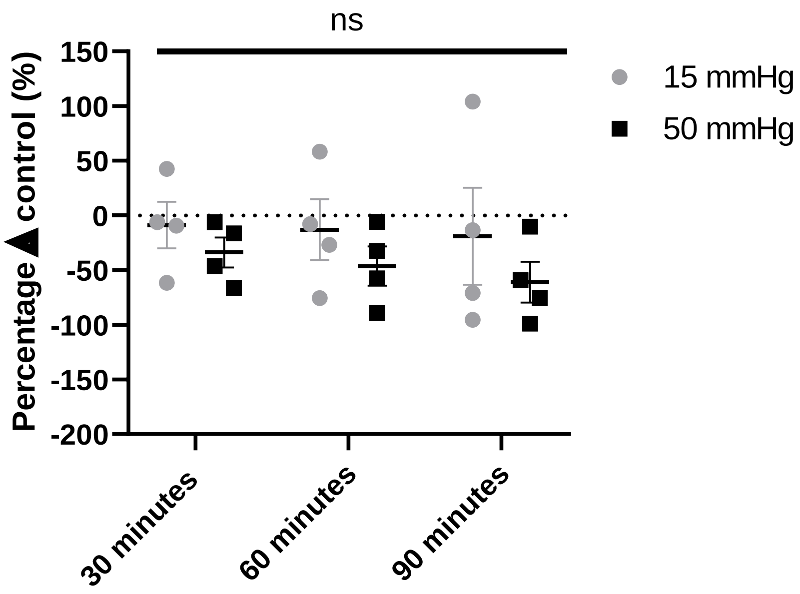


Figure 5.8: Peak firing at 15 mmHg and 50 mmHg showed no significant difference between the early and later time points (p = 0.3429; *n* = 4; two-way ANOVA).

The effect of increasing concentrations of BoNT/A (0) on bladder mechanosensitivity

BoNT/A (0) induced inhibition of bladder sensation appeared to be dose dependent, as preparations treated with 3 pM (*n* = 8) exhibited a more potent inhibition of distension induced afferent firing than 1 fM (*n* = 4) treated preparations (figure 5.9).

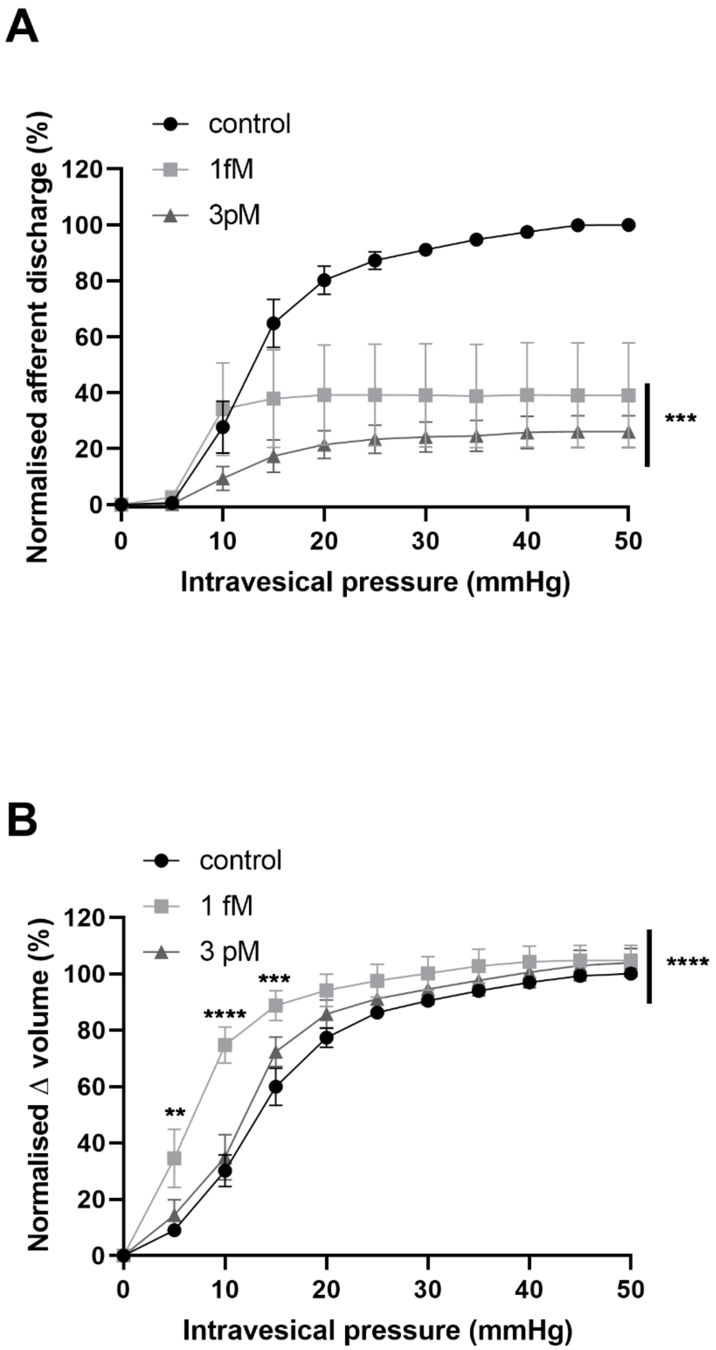


Figure 5.9: A comparison of the inhibitory effect induced by 1 fM and 3 pM BoNT/A (0). **A)** 3 pM BoNT/A (0) inhibited nerve firing more potently than 1 fM (p = 0.0008; two-way ANOVA). **B)** 1fM BoNT/A (0) significantly increased compliance compared to 3 pM BoNT/A (0), especially at lower pressures (up to 15 mmHg) (p<0.0001; *n* = 4; two-way ANOVA with Bonferroni's post-test).

5.2 The effect of BoNT/A (0) on capsaicin induced afferent firing

To further characterise the effect of BoNT/A (0) on sensory signalling, the effect of BoNT/A (0) pretreatment on capsaicin induced neuronal excitation was investigated. After distension of the bladder to 20 mmHg, the tap was closed, in order to capture any contractions induced by extraluminal capsaicin. Application of 1 µM capsaicin led to a spike in neural activity, due to the activation of TRPV1 channels (figure 5.10A). There was no significant difference in the capsaicin response in BoNT/A (0) pretreated preparations, as compared to control (figure 5.10B; p= 0.8349; two-way ANOVA). This was also evident in the AUC analysis which showed no difference (figure 5.10C; p= 0.7548; T-test), nor was there a difference in the peak firing induced by capsaicin (figure 5.10D; p= 0.8684; T-test). Interestingly, while the neural response to capsaicin was similar in the control and BoNT/A (0) pretreated preparations, this was not the case for the contractile response which was lost (figure 5.10E; p= 0.0264).

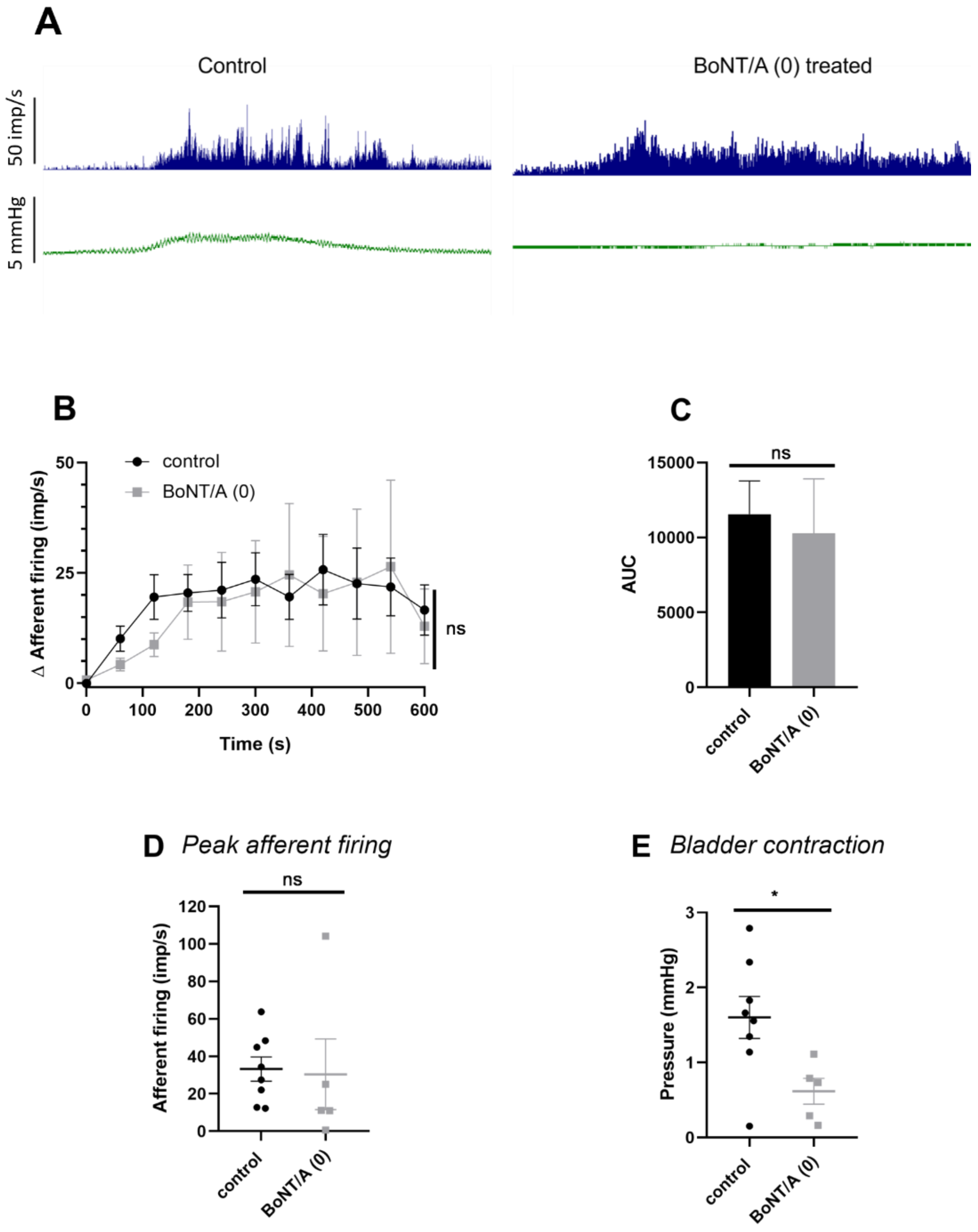


Figure 5.10 The effect of BoNT/A (0) pretreatment on the response to capsaicin. **A)** A representative figure showing the nerve response and intravesical pressure change in control (left) and BoNT/A (0) pretreated (right) preparations. **B)** There was no difference between the capsaicin induced nerve responses between

the control (*n* = 8) and the BoNT/A (0) pretreated (*n* = 6) preparations (p = 0.8349; two-way ANOVA). **C)** The AUC analysis also showed no difference between preparations (p = 0.7548; t-test). **D)** Peak firing elicited by capsaicin treatment was not different between preparations (p = 0.8684; t-test). **E)** Peak contraction elicited by capsaicin treatment was significantly lower in BoNT/A (0) treated preparations than control preparations (p = 0.0264; t-test).

5.3 Effect of BoNT/A (0) on bladder accommodation

In order to test the effect of BoNT/A (0) on bladder accommodation, the bladders were filled to a pressure of 20 mmHg, at which point the intraluminal pump was closed and the volume within was maintained (figure 5.11A). Gradually, the intravesical pressure decreased as the bladder accommodated to this volume. All the preparations tested exhibited a decrease in intravesical pressure, however this was larger in the BoNT/A (0) pretreated preparations ($p = 0.0358$; $n = 5$; figure 5.11B). The filling to 20 mmHg is also coupled with afferent excitation, this appeared to be significantly lower in BoNT/A (0) pretreated preparations ($p<0.0001$; figure 5.11C), however the AUC analysis did not reach significance ($p=0.061$; figure 5.11D). This data suggests BoNT/A (0) may interfere with the detrusor muscle or lamina propria layers and their ability to maintain intravesical pressure, as well as inhibiting mechanosensitivity.

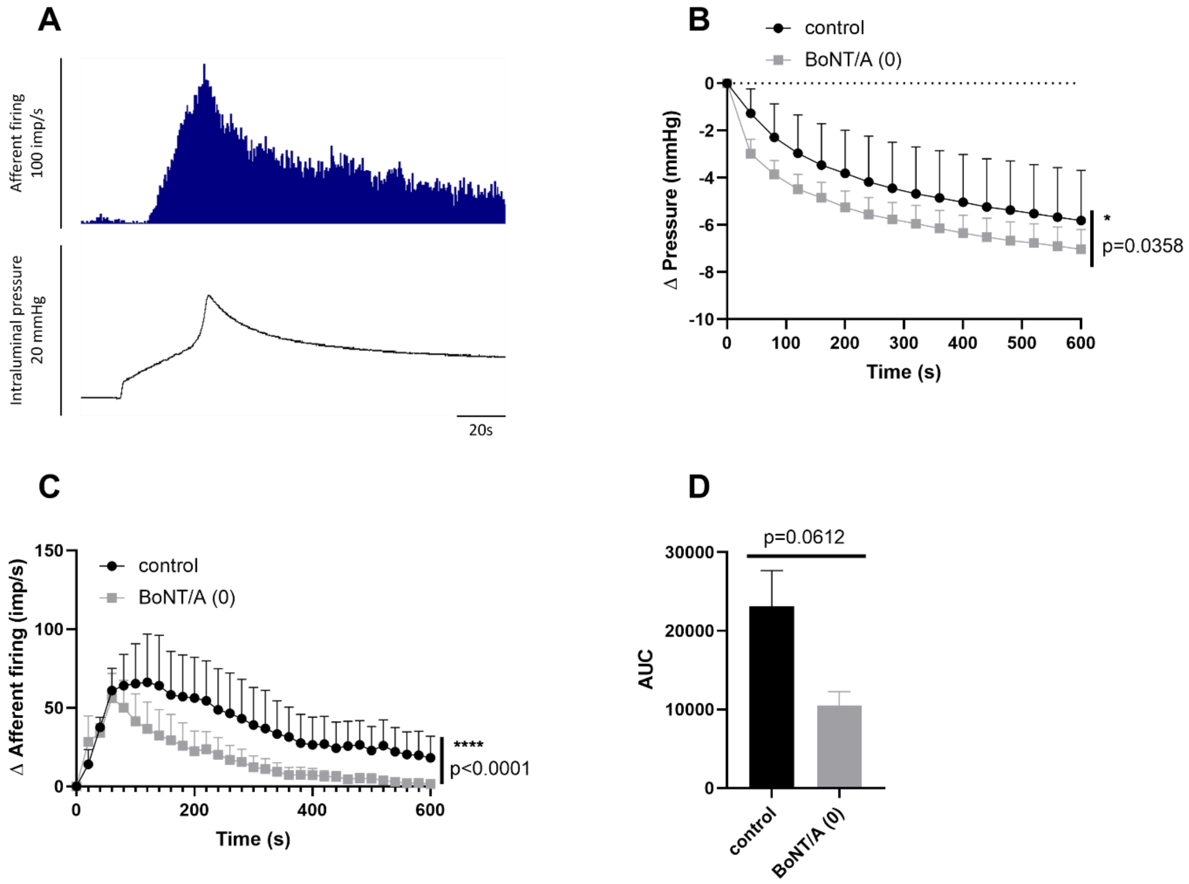


Figure 5.11 The effect of BoNT/A (0) on bladder accommodation. **A)** A representative figure showing the effect of filling the bladder to 20 mmHg and stopping the pump, which leads to accommodation of intravesical volume. **B)** BoNT/A (0) pretreated preparations showed a significantly larger drop in pressure after bladder filling was stopped (p = 0.0358; *n* = 5; two-way ANOVA) compared to control preparations (*n* = 8). **C)** Afferent firing to 20 mmHg was significantly lower in BoNT/A (0) pretreated preparations than control preparations (p <0.0001; two-way ANOVA). **D)** This data represented as AUC did not reach significance (p = 0.061; one-way ANOVA).

5.4 Effect of LC/A (0) on bladder mechanosensitivity

To ensure the potent inhibition induced by BoNT/A (0) depended on the mutation in the light chain, an LC/A (0) construct that contained the same mutation was assessed. Application of 3.6 pM LC/A (0) significantly reduced bladder mechanosensitivity (figure 5.12A), as after 90 minutes post-treatment, 58.97% (+/- 12.58%) of distension induced nerve firing remained ($p<0.0001$; figure 5.12A; $n = 5$; two-way ANOVA). Peak firing at 15 and 50 mmHg was significantly reduced in a time dependent manner following treatment ($p = 0.0334$; figure 5.13), specifically at the 60 minute time point which suggests LC/A (0) inhibited units responsive at 50 mmHg significantly more than nerve units responsive at 15 mmHg ($p = 0.0355$; figure 5.13).

Bladder compliance was significantly increased following LC/A (0), as higher volumes were necessary to reach the 50 mmHg pressure ($p<0.0001$; $n = 5$; figure 5.12B). This increase in pressure-volume relationship was seen as early as 30 minutes after LC/A (0) treatment ($p = 0.0098$; figure 5.13B; one-way ANOVA).

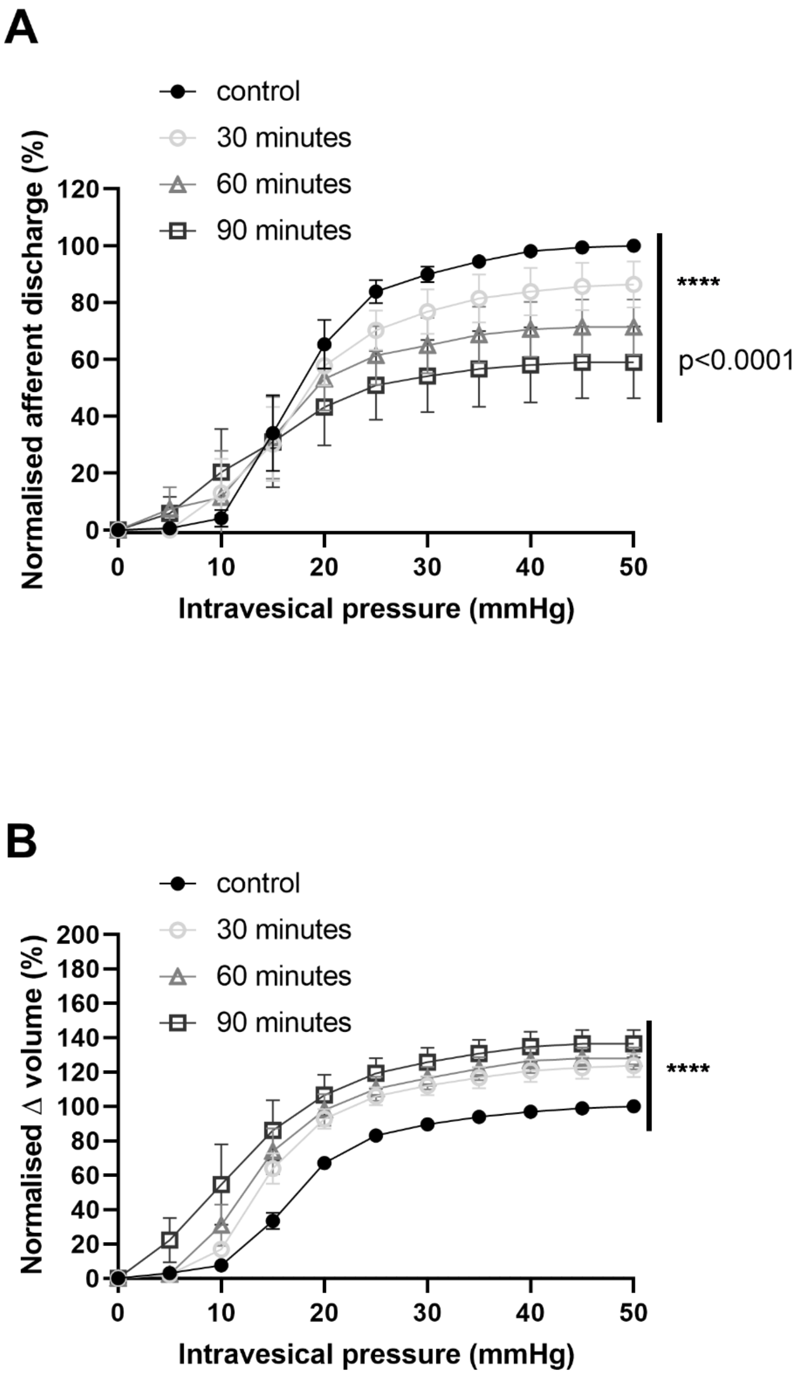


Figure 5.12: The effect of LC/A (0) on distension induced afferent firing. **A)** Afferent responses to distension were reduced following LC/A (0) treatment ($p<0.0001$; n = 5; two-way ANOVA). **B)** The pressure-volume relationship was significantly increased after intravesical LC/A (0) compared to control ($p < 0.0001$; n = 5; two-way ANOVA).

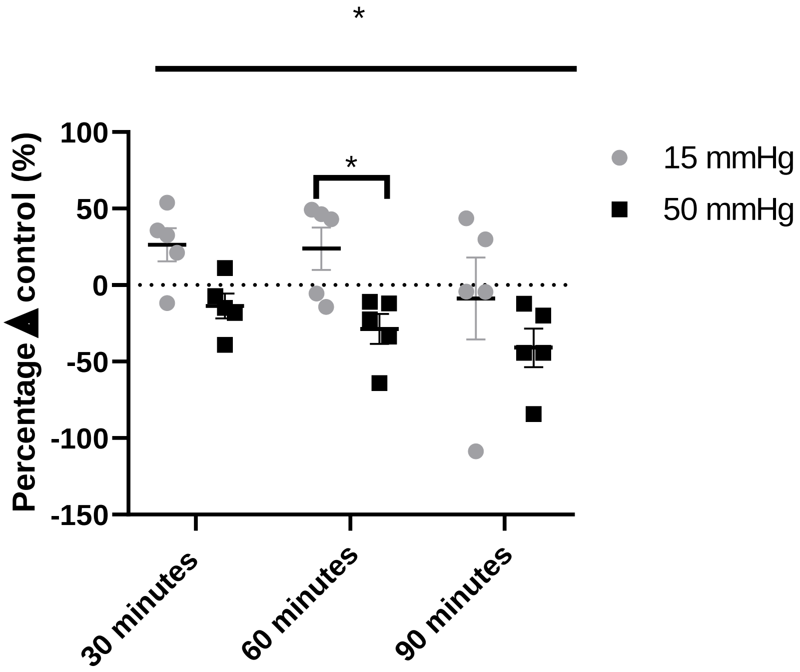


Figure 5.13: Peak firing at 15 mmHg and 50 mmHg was significantly reduced in a time dependent manner (p = 0.0334; *n* = 5; two-way ANOVA). Bonferroni's post-hoc test revealed a significant difference in the inhibition induced by LC/A (0) at 60 minutes (p = 0.0355).

5.5 The effect of nitric oxide synthase inhibitor L-NAME on BoNT/A (0) induced inhibition of afferent firing

Previous studies have shown increases in nitric oxide (NO) release following treatment with native BoNT/A (Collins et al., 2013; Smith et al., 2008), however the mechanism behind this effect is unclear. This is unlikely to be due to cleavage of SNAP-25, as NO synthase is not involved in SNARE processes or vesicular release pathways. To investigate the role of the NO pathway on the induction of BoNT/A (0) mediated afferent inhibition, the non-selective NO synthase inhibitor NG-Nitro-L-arginine methyl ester hydrochloride (L-NAME, Tocris) was co-applied with BoNT/A (0). L-NAME alone had no effect on distension induced afferent firing (figure 5.14A; p= 0.4750) nor on bladder compliance (figure 5.14B; p = 0.3325).

When 1 mM L-NAME was co-applied with BoNT/A (0), the potent inhibitory effect of BoNT/A (0) was lost, leading to a slight hyperactivity which was not significant (figure 5.15A; p = 0.3881). There appeared to be significant increased bladder compliance over 90 minutes (figure 5.15B; p = 0.0078). This data suggests the involvement of nitric oxide signalling in the potent inhibition induced by BoNT/A (0).

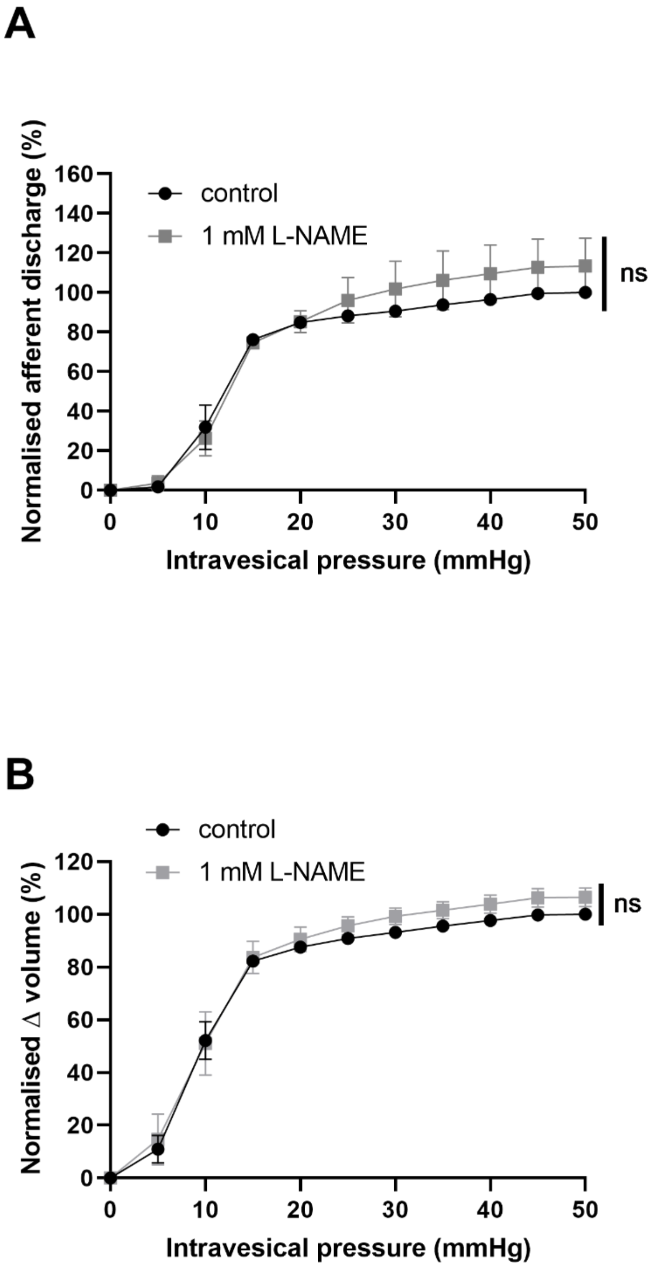


Figure 5.14: L-NAME treatment alone did not alter distension induced afferent firing. **A)** The afferent response to intravesical L-NAME was not different to that of control (p = 0.475; *n* = 5; two-way ANOVA). **B)** The pressure-volume relationship was not altered by intravesical L-NAME (p = 0.3325; *n* = 5; two-way ANOVA).

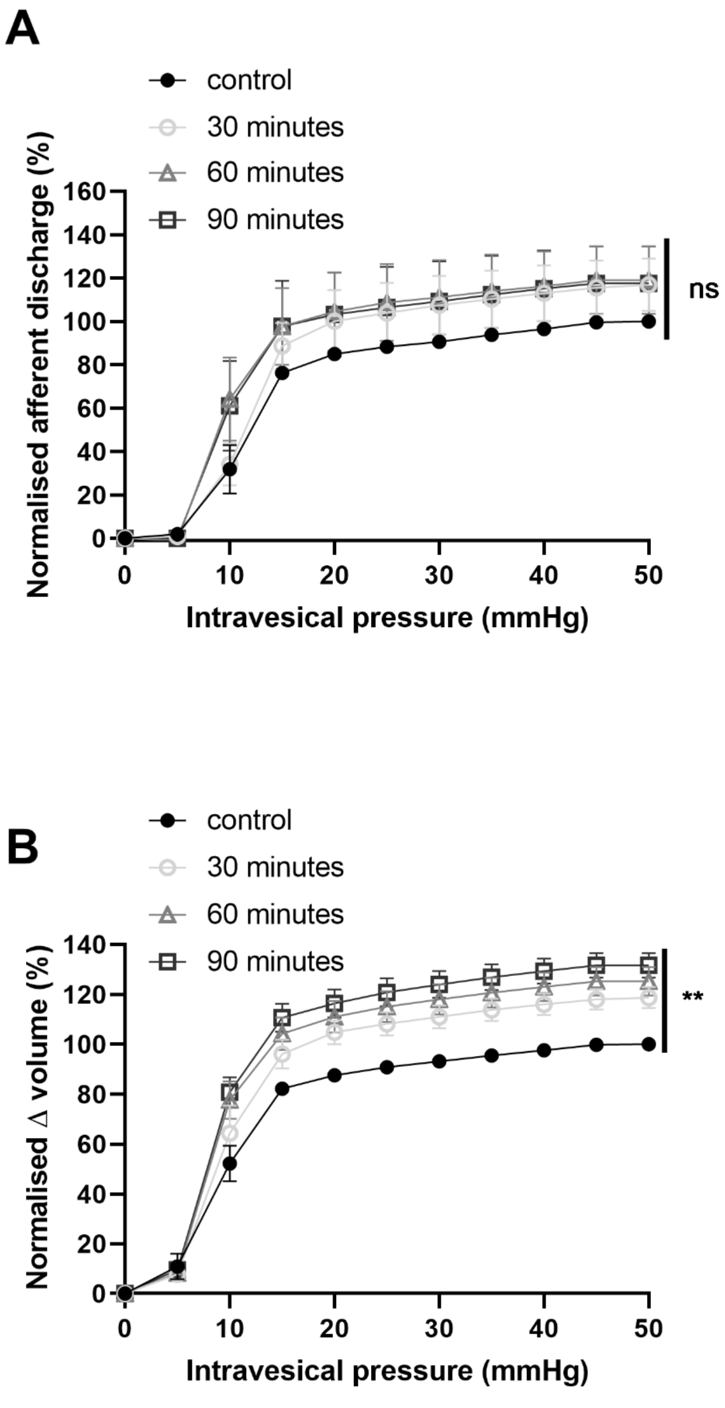


Figure 5.15: co-application of BoNT/A (0) and L-NAME reversed the BoNT/A (0) induced afferent inhibition.

A) Afferent responses to distension following L-NAME + BoNT/A (0) treatment were not significantly different as compared to control (p = 0.3881; *n* = 5; two-way ANOVA). **B)** The pressure-volume relationship was significantly increased by intravesical L-NAME + BoNT/A (0) (p= 0.0078; *n* = 5; two-way ANOVA).

5.6 The effect of purinergic antagonist TNP-ATP on BoNT/A (0) induced afferent inhibition

BoNT/A induced inhibition of urothelial release of ATP has been shown repeatedly in the literature (Collins et al., 2013; Hanna-Mitchell et al., 2015; Khera et al., 2004; Smith et al., 2005, 2008). This could be due to cleavage of SNAP-25, as there is evidence ATP is released through vesicles, however other mechanisms of release such as pannexin and connexin hemichannels have also been proposed (McLatchie et al., 2014). To explore the role of the purinergic pathway on the BoNT/A (0) mediated afferent inhibition, the P2X receptor antagonist 2',3'-O-(2,4,6-Trinitrophenyl)adenosine-5'-triphosphate tetra(triethylammonium) salt (TNP-ATP, Tocris) was co-applied with BoNT/A (0).

When 30 µM TNP-ATP was applied intravesically alone, it had no effect on distension induced afferent firing (figure 5.16A; *n* = 6; p = 0.4396) nor on bladder compliance (figure 5.16B; *n* = 6; p = 0.2868). However, when co-applied with BoNT/A (0), there was no significant difference in distension induced afferent firing (figure 5.17A; *n* = 6; p = 0.1172), suggesting TNP-ATP was able to modulate the inhibitory action of BoNT/A (0). There also appeared to be significantly increased bladder compliance following the 90 minutes (figure 5.17B; *n* = 6; p <0.0001).

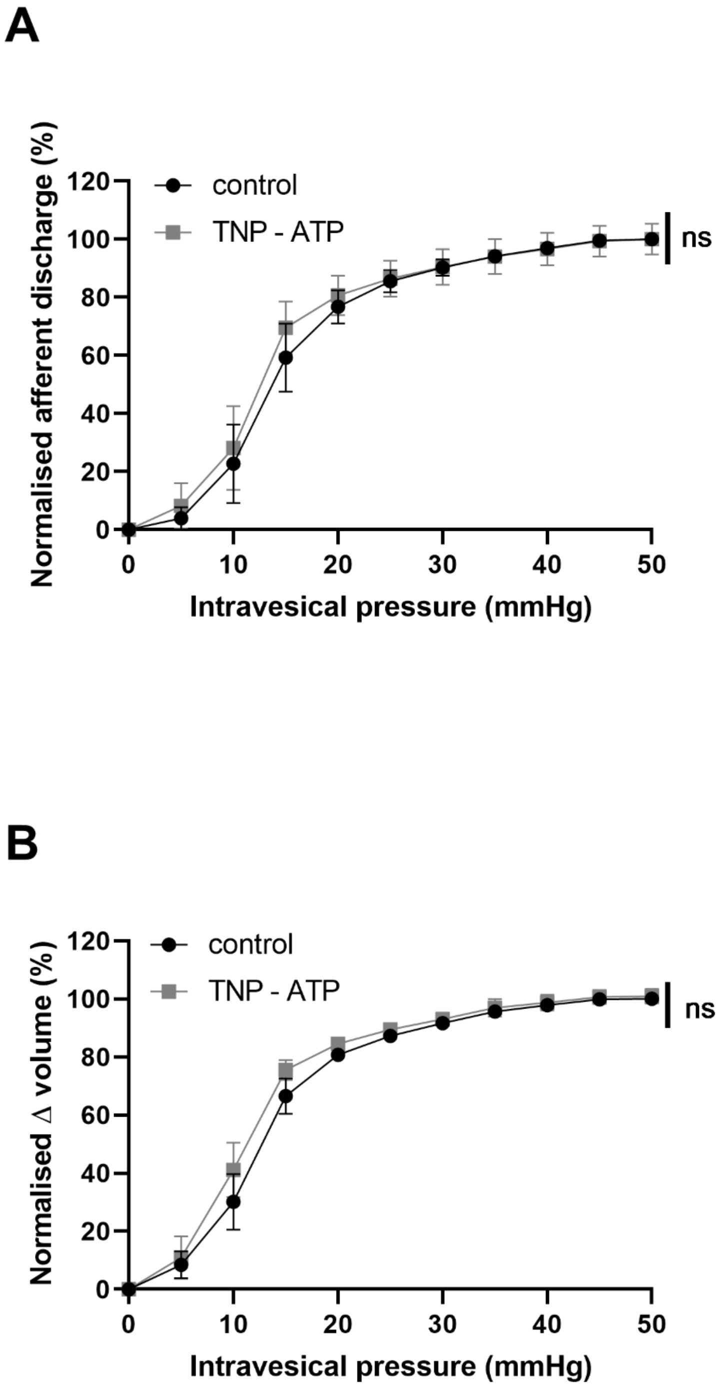


Figure 5.16: The effect of intravesical TNP-ATP on bladder mechanosensitivity, **A)** The afferent response to intravesical TNP-ATP was not different to that of control (p = 0.4396; *n* = 6; two-way ANOVA)**. B)** The

pressure-volume relationship was not altered by intravesical TNP-ATP (p = 0.2868; *n* = 6; two-way ANOVA).

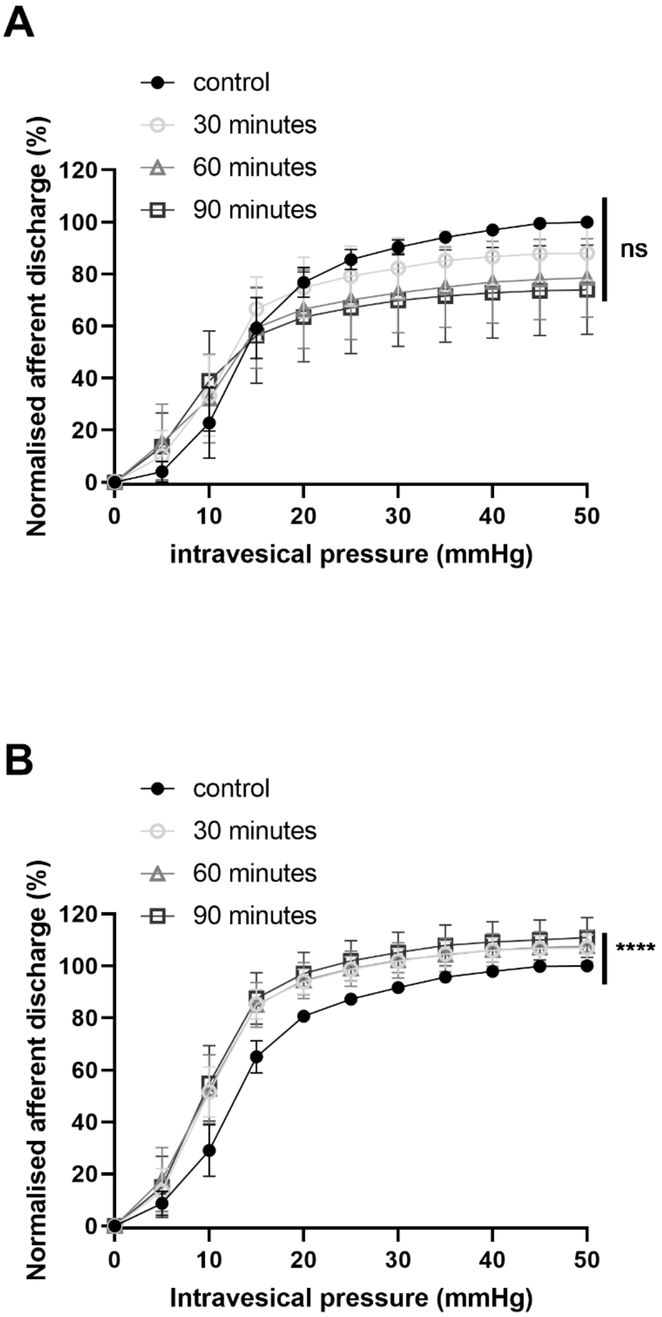


Figure 5.17: The effect of coapplication of TNP-ATP and BoNT/A (0) on bladder mechanosensitvity**. A)** Afferent responses to distension following TNP-ATP + BoNT/A (0) treatment were not significantly different

as compared to control (p = 0.1172; *n* = 6; two-way ANOVA). **B)** The pressure-volume relationship was significantly increased by intravesical TNP-ATP + BoNT/A (0) (p<0.0001; *n* = 6; two-way ANOVA). D) this was also reflected in the AUC analysis (p = 0.0309; *n* = 6; one-way ANOVA).

DISCUSSION

The main findings described in this chapter are

- A catalytically inactive construct of BoNT/A, named BoNT/A (0), significantly inhibited bladder mechanosensation. The degree of inhibition was greater than that observed with the catalytically active construct (see chapter three).
- Potent inhibition was also found using the LC/A (0), suggesting this response was not receptor dependent, supporting findings in chapter three.
- Chemosensation did not appear to be altered by BoNT/A (0) pretreatment, as the neural capsaicin response was not different to control preparations. However, the concomitant contraction seen in control preparations was not observed in BoNT/A (0) pretreated preparations
- The BoNT/A (0) induced inhibition of distension-induced afferent signalling was reversed by inhibiting nitrergic and purinergic pathways.

The aim of this chapter was to characterise a novel BoNT/A construct unable to cleave SNAP-25, with the goal of better understanding the underlying mechanisms behind BoNT/A induced inhibition of afferent firing. As shown in chapter three, Dysport and other BoNT/A constructs were able to significantly reduce distension induced firing, however, there appeared to be no presence of cleaved SNAP-25 in the bladder wall. This necessitated a more direct approach to investigating the role of SNAP-25 in the induction of sensory inhibition, which was conducted using a BoNT/A construct with a catalytically inactive light chain.

Catalytically inactive BoNT/A potently inhibited bladder mechanosensation

A mutation in the HEXXH region in the light chain is responsible for the loss of SNAP-25 cleavage and resultant paralytic activity of BoNT/A (0) (see methods figure 5.2). The data described in this chapter show BoNT/A (0) significantly inhibited bladder mechanosensation, despite its non-functional light chain. While this data proves SNAP-25 cleavage is not necessary to reduce distension induced afferent firing, it is more striking that BoNT/A (0) inhibited afferent firing more potently than every form of catalytically active BoNT/A investigated in chapter three. BoNT/A (0) was effective at extremely low concentrations, treatment of 1 fM BoNT/A (0) reduced peak afferent firing to 38.9% (+/-18.8%; figure 5.7A) after 90 minutes. It is unclear how BoNT/A (0) managed to induce such significant inhibition at low concentrations, and which cell type it targeted to induce this effect.

Previous studies showing reduction in sensory signalling following BoNT/A treatment suggest SNAP-25 is responsible for this effect, as cleavage may stop the release of neuropeptides from the sensory nerve terminal. This may interrupt the communication between afferent nerves and nearby cell types and perhaps stop the propagation of sensory signals. However, it remains unclear whether SNAP-25 cleavage directly inhibits afferent signalling, and whether this process is responsible for therapeutic effects in clinical studies. In fact, there is evidence that a therapeutic effect may be achieved without SNAP-25 cleavage, Liu *et al* (2015) reported that bladder tissue of patients who received intravesical liposome encapsulated BoNT/A did not show cleaved SNAP-25 immunoreactivity, despite the significant reduction of urgency as well as reduced expression of P2X3 receptors in the urothelium (Liu et al., 2015). This data introduces the possibility of SNAP-25 independent function of BoNT/A in the inhibition of sensory signals, which is supported by the IHC findings in chapter three.

The mechanisms underlying the potent afferent inhibition induced by BoNT/A (0) reported in this chapter are unclear, as all other studies conducted using catalytically inactive forms of BoNT/A have shown no effect (Baskaran et al., 2013; Ravichandran et al., 2015; Yang et al., 2008). Baskaran *et al* (2013) characterised the deactivated recombinant BoNT/A (drBoNT/A) and its effect on the mouse toe spread reflex and acetylcholine release. They found that the toe spread reflex was not affected by drBoNT/A, in contrast to the inhibition induced by the wild type active BoNT/A (Baskaran et al., 2013). They also found no effect on spontaneous or nerve evoked acetylcholine release which was blocked by wild type BoNT/A (Baskaran et al., 2013). This is despite their finding that drBoNT/A was internalized and localised within the motor neuron at its expected site of action (Baskaran et al., 2013). These data suggest inactive BoNT/A can enter cells as normal and can gain access to SNAP-25, however it cannot disrupt its function, as SNAP-25 retained the ability to facilitate exocytosis and induce muscle contraction. These findings are supported by the hemidiaphragm experiment showing the lack of paralytic activity of BoNT/A (0) (see figure 5.3), which shows muscle contractility remained on par with that of the vehicle treated preparations. These results collectively confirm that mutation of the HEXXH motif in the BoNT/A (0) light chain results in complete loss of neuroparalytic activity and has no effect on neurotransmitter release from motor nerves, while data in this chapter shows BoNT/A (0) induces potent inhibition of sensory neurotransmission. The potential mechanisms underlying this effect are discussed below.

One of the more intuitive explanations of the potent sensory inhibition induced by BoNT/A (0) was based on its striking structural similarity to active BoNT/A, that it may retain the ability to target and bind to SNAP-25 despite its inability to cleave it. This may lead to the interruption of the formation of the SNARE complex necessary to facilitate exocytosis, and potentially indirectly inhibit release of neurotransmitters. This potential mechanism is inconsistent with the *in vivo, ex vivo* and *in vitro* data discussed above, which show the SNARE complex remains intact and functional following treatment with BoNT/A (0) (Baskaran et al., 2013). This mechanism does not explain why BoNT/A (0) inhibited sensory signalling significantly more potently than that induced by the multiple forms of catalytically active BoNT/A tested in chapter three. It is curious that while Dysport has been widely shown to cleave SNAP-25 (Oliveira et al., 2017), there was no evidence of the cleaved form in the IHC study, which suggests cleavage of SNAP-25 may not be necessary to induce afferent inhibition. This reveals an interesting problem where neither active nor inactive BoNT/A appear to cleave SNAP-25 in the present preparation yet have vastly different effects on bladder afferent signalling, with the inactive form having a stronger effect on inhibiting afferent signalling. The only difference between the compounds remains the two amino acid substitutions in the light chain which endows BoNT/A (0) with the significantly higher biological activity, however the potential effects these substitutions have on the structure and function of BoNT/A (0) remain unclear.

Previous studies have found changes in gene expression in a variety of cell types following treatment with BoNT/A, including receptors such as TRP channels (Cao et al., 2017; Fan et al., 2017; Shimizu et al., 2012), purinergic receptors (Yang et al., 2022) and sodium channels (Yang et al., 2016). The mechanisms underlying the transcriptional changes induced by BoNT/A are unclear, the cells may be adapting to the SNAP-25 cleavage mediated inhibition of exocytosis which may lead to an aggregation of receptor-containing vesicles intended to be expressed on the plasma membrane. This could stimulate the initiation of a negative feedback process to stop the continued transcription and translation of these receptors. To investigate whether an inactive form of BoNT/A may also affect gene expression, Scherf *et al* (2014) exposed cells to both active and inactive BoNT/A. Interestingly, they found that the expression of the vast majority of genes tested were similarly altered by active and inactive BoNT/A, with a small number of genes differentially regulated (Scherf et al., 2014). An example of genes highly upregulated by both active and inactive BoNT/A were genes responsible for Ca^{2+} channel regulation, which the authors suggest may be due to interruption or cleavage of the SNAP-25 protein, as it has complex interactions with Ca^{2+} channels (Scherf et al., 2014). While the inactive BoNT/A molecule may interact with SNAP-25, it is very unlikely to inhibit its activity to a level that is comparable to the active BoNT/A, which makes it difficult to reconcile transcriptional regulation being altered by active and inactive BoNT/A through the same mechanism. It is also unclear how the two compounds may act through different mechanisms yet reach the same goal of altering the transcriptional regulation of largely the same genes. These results suggest active and active BoNT/A may work through a common, SNAP-25 independent mechanism. In the context of the present study, while it may be possible that BoNT/A and BoNT/A (0) can inhibit afferent signalling by modulating the transcriptional regulation of mechanosensitive receptors, the differences in time courses of the experiments in the present study and that of previous studies make this unlikely. For example, Scherf *et al* (2014) found little changes in transcriptional regulation two days after exposure to active and inactive BoNT/A, while two weeks after exposure they found hundreds of genes differentially expressed compared to control preparations. This suggests longer incubation times are necessary to see changes in transcription, which may rule it out as a potential mechanism of action of BoNT/A (0).

One of the roles of the BoNT light chain is to localise to the correct intracellular compartment to find and cleave its SNARE target, as Fernández-Salas *et al* (2004) found LC/A present along the plasma membrane, the light chain of BoNT/B (LC/B) throughout the cell and the BoNT/E light chain (LC/E) within the cytosol (Fernández-Salas et al., 2004). If the LC/A (0) localised to a different intracellular compartment such as the cytosol, it may have interacted with other proteins that it may not have encountered without its mutation. This has been shown to not be the case by Fernández-Salas *et al* (2004) who tested the efficacy of a catalytically inactive light chain mutant with substitutions in the same region as BoNT/A (0) (H227Y, see methods), finding that it retained plasma membrane localisation (Fernández-Salas et al., 2004). This narrows the potential intracellular site of action of BoNT/A (0), suggesting it may be acting on proteins present close to the plasma membrane to induce its potent inhibitory action on sensory signalling.

There is a body of literature describing SNAP-25 independent functions of active BoNT/A that may provide additional clues as to how BoNT/A (0) may be working. Investigators have shown BoNT/A may inhibit exocytosis indirectly by acting on the Rho GTPases in the phospholipase A2 pathway (Ishida et al., 2004; Ray et al., 1993, 1999; Zhang et al., 2013). Rho GTPases are activated by a range of receptors, such as G protein coupled receptors (GPCRs) and ion channels to perform a wide range of cellular functions, from membrane trafficking, regulation of transcription and cellular growth (Aelst et al., 1997; Bhattacharya et al., 2004; Pochynyuk et al., 2007). This is achieved through the switch function of GTPases, which alternate between the active GTP bound state and the inactive GDP bound state. Ishida *et al* (2004) found active BoNT/A inhibited ACh release by accelerating the degradation of the Rho GTPase RhoB by ubiquitin (Ishida et al., 2004). Active BoNT/A dependent upregulation of Rho GTPases has been reported previously, providing further evidence of its modulation of this pathway (Park et al., 2016). A recent study looking at the function of BoNT/A (0) found it acting on Rac1, also part of the Rho GTPase family (Valois et al., 2021), however, they found that activation of Rac1 was driven by the HC_c domain. If the HC/A is responsible for the interaction with Rac1, it is difficult to reconcile this with data in chapter three which shows the HC/A domain had no effect on afferent mechanosensitivity in the bladder. In fact, the data in chapters three and five shows that the domain responsible for the afferent inhibition was the light chain. These findings point to the biological function of both active and inactive BoNT/A being dependent on the light chain domain, and any SNAP-25 independent functions must show interaction with the light chain.

While the data in this chapter shows direct action of BoNT/A (0) and LC/A (0) on bladder mechanosensation, further studies are needed to clarify the exact mechanism of action that leads to the potent inhibition of sensory signalling, as well as reconcile the discrepancy found in this thesis between the biological effects of active and inactive BoNT/A.

BoNT/A (0) pretreatment did not appear to affect afferent excitation induced by capsaicin, however the concomitant contractions were not present

Capsaicin is a chemical agonist of the multimodal transient receptor potential vanilloid (TRPV1) receptor, which is also activated by heat, mechanical stimulation, and acid (Elokely et al., 2016).

The afferents innervating the bladder wall express a variety of sensory receptors at their terminals, responsive to mechanical and chemical stimuli. While BoNT/A (0) application had been shown to cause a significant decline in the mechanosensitivity of the afferent nerves, it remained unclear whether detection of chemical stimuli was also affected. The rationale behind investigating whether capsaicin sensitivity was altered by BoNT/A (0) pretreatment was to better understand if afferent excitation was globally reduced, or if the potent inhibition of distension induced firing was due to targeting of specific receptors or signalling pathways. The multimodality of TRPV1 activation made it an attractive receptor to begin answering this question.

In control preparations, application of 1 µM capsaicin led to afferent excitation and simultaneous contraction (figure 5.10). Interestingly, this response pattern was not seen in the BoNT/A (0) pretreated preparations, where afferent excitation happened alone without detrusor contraction. In a study using catalytically active BoNT/A, Atiemo *et al* (2005) applied intravesical capsaicin and ATP into rat bladders in an *in vivo* preparation and found pretreatment with BoNT/A significantly reduced the contraction associated with ATP while the reduction in the capsaicin response was not significant (Atiemo et al., 2005). In this study, intravesical application of ATP and capsaicin were used as a model of detrusor overactivity, and the authors did not seem to point out a specific mechanism by which BoNT/A reduces contractility induced by ATP. As the injections were applied prior to the cystometry experiment, BoNT/A may have reduced purinergic receptor expression, or somehow altered sensory responses to ATP. However, the *in vivo* nature of their preparation does not preclude involvement of the efferent limb, and reduced contractility may be due to some paralysis of the detrusor muscle. In the *ex vivo* preparation, the efferent neurotransmission is non-functional, which suggests any contractions in response to capsaicin are most likely due to release of neuropeptides either from the afferent nerves, the urothelium, suburothelial cells or smooth muscle cells upon activation of TRPV1.

This data provides an interesting perspective of BoNT/A (0) dependent modulation of bladder sensory signalling. Although the mechanism underlying its inhibition of mechanosensation is unknown, the capsaicin data suggests BoNT/A (0) may not render the afferent terminal unable to detect sensory signals. Why chemical agonism of TRPV1 elicits an afferent response however mechanical stimulation is inhibited is unclear. If BoNT/A (0) specifically inhibits mechanosensory neurotransmission instead of sensory signalling more globally, it may be suggested that stretch sensitive receptors are targeted directly, such as ENaC, other TRPs such as TRPV4 and Piezo channels. Due to the novelty of the BoNT/A (0) molecule, there is currently no evidence pointing to its modulation of stretch sensitive receptors, which may be explored in detail in future investigations.

The ability of capsaicin to elicit bladder contraction is well known, Santicioli *et al* (1986) showed capsaicin induced contractility depended on intact afferent neurotransmission, as this effect was abolished by denervation (Santicioli et al., 1986). Maggi *et al* (1991) found that capsaicin induced activation of afferent nerves led to release of tachykinins such as substance P and neurokinin A, which subsequently acted on NK2 receptors on detrusor smooth muscle (Maggi et al., 1991). As discussed above, it is unlikely that catalytically inactive BoNT/A may interact with SNAP-25 in a way that it might affect neurotransmitter release, however, it is also unclear how else capsaicin induced detrusor contractility may be inhibited. Further studies involving direct recording of tachykinin release from afferent terminals may provide an answer.

The LC/A (0) protein significantly inhibited bladder mechanosensitivity, more potently than active LC/A.

While the double receptor mechanism of BoNT/A entry has been well described in the literature, data presented in this thesis (see chapter three) showed the LC/A was able to enter cells and induce afferent inhibition. This was unexpected as the exquisitely sensitive targeting of cholinergic nerves underlies the neuroparalytic action of BoNT/A. However, due to lack of consensus in the literature regarding expression of the SV2 receptor in urothelial cells and afferent nerve terminals, it was unclear whether this entry mechanism was utilised by BoNT/A. In chapter three, the catalytically active LC/A significantly inhibited distension induced afferent firing, which proved the double-receptor mechanism was not necessary in modulating sensory signalling.

In the present chapter, the catalytically inactive LC/A (0) was tested using an identical protocol, the results revealed significant inhibition of distension induced afferent firing (figure 5.12). The increased potency of LC/A (0) compared to the active LC/A follows the same pattern as that of the full length BoNT/A constructs, which provides further evidence to the lack of involvement of the double receptor mechanism in the entry of the BoNT/A light chain. These data suggest the light chain is the domain necessary to induce sensory inhibition in both the catalytically active and inactive constructs. While the mechanism of sensory inhibition is not known, the mutation in the HEXHH motif that renders BoNT/A (0) and LC/A (0) inactive may have led to structural changes that increased the affinity to its target.

It remains unclear which entry mechanism is utilised by LC/A and LC/A (0) to enter cells of interest. One potential mechanism is entering through the discoid fusiform vesicles (DFVs) formed in umbrella cells as the bladder is emptying. It is less clear how the light chain may translocate out of the DFVs to enter the cytoplasm, as it lacks the Hn translocation domain responsible for creating the pore in the endocytic vesicle to allow the light chain to cross into the cytoplasm. Further studies are necessary to better understand how the light chain may enter cells to inhibit afferent firing.

In the *ex vivo* bladder electrophysiology preparation, intravesical pressure is recorded concomitantly with afferent firing, and this can be used to infer compliance and any effects the drugs of interest are having on compliance. In figure 5.12B, dramatic increases in the pressure-volume relationship were observed over 90 minutes following LC/A (0) treatment, which was unexpected as the full length BoNT/A (0) had no effect on compliance (figure 5.5B). The difference in result may be due to the size of the protein, as BoNT/A (0) is 150 kDa while the LC/A (0) is much smaller at 50 kDa, which may have improved penetrance further into the bladder wall. Previous studies have shown active BoNT/A to increase expression of collagen when injected into the skin (Oh et al., 2012), which may lead to increased collagen deposition. If the smaller size of the LC/A (0) lends itself to increased penetrance into the bladder wall, it may travel into deeper layers to deposit collagen into the lamina propria or detrusor, potentially leading to increased accommodation and compliance of the bladder.

The effect of purinergic and nitrergic antagonists on BoNT/A (0) induced afferent inhibition

As BoNT/A and its catalytically inactive counterpart are near identical in structure apart from two amino acid substitutions in the light chain, it is reasonable to assume any non-SNAP-25 dependent effects may be shared between them. Previous studies have found alterations in bladder signalling pathways following BoNT/A treatment, Collins *et al* (2013) reported decreased ATP release and increased nitric oxide (NO) release after intravesical application of BoNT/A (Collins et al., 2013). In a rat model of chronic spinal cord injury, Smith *et al* (2008) found that intravesical injection of BoNT/A reduced evoked ATP release and increased NO release, normalising both to control levels (Smith et al., 2008). Although ATP release has been shown to be partly mediated through exocytotic mechanisms controlled by SNARE proteins, other mechanisms of release have been reported including via pannexin and connexin hemichannels (Ransford et al., 2009; Timóteo et al., 2014). While NO release is completely independent from SNARE proteins, instead produced by nitric oxide synthases. As ATP release is partially SNAP-25 independent and NO release is wholly SNAP-25 independent, the aim of these experiments were to better understand if BoNT/A (0) achieves the potent inhibition of bladder afferent signalling through modulation of these signalling pathways.

In the present study, inhibition of nitric oxide synthase using L-NAME had no effect on distension induced afferent firing alone (figure 5.14). This finding is supported by Yu *et al* (2013) who also found L-NAME did not affect basal mechanosensitive firing, although in their study they used a concentration of 20 mM, much higher than that of the present study (1mM) (Yu & de Groat, 2013). Previous studies have also shown hyperexcitability in afferent responses following L-NAME treatment, for example, Aizawa *et al* (2011) found that intravesical instillation of L-NAME caused significantly increased responses of both Aδ and C fibres, although their study was *in vivo* (Aizawa et al., 2011). These data suggest the role of NO in normal bladder sensation is still not completely clear. Interestingly, when co-applied with BoNT/A (0), the L-NAME caused a reversal of the potent inhibition seen earlier in this chapter, suggesting involvement of the nitric oxide pathway in the mechanism of action of BoNT/A (0) (figure 5.15). As L-NAME is a non-selective nitric oxide synthase (NOS) inhibitor, it is not possible to deduce which subtype of NOS is affected by BoNT/A (0), which would allow us to pinpoint which cell type could be the site of action. For example, it is possible that BoNT/A (0) acts solely in the urothelium and may lead to the activation of endothelial, neuronal or inducible NOS, all of which have been found to be expressed in the urothelium (Birder et al., 2002; Gillespie et al., 2005). As a result, the increased concentration of NO may act on the primary afferent nerve terminals to attenuate mechanosensation, in an autocrine fashion to reduce urothelial release of ATP or it can act on the suburothelial interstitial cells, which express cGMP in response to NO (Gillespie et al., 2005; Smet et al., 1996). Elevated levels of cGMP may also reduce afferent excitability, as shown by Yoshimura *et al* (2001) who found reduced activity of N-type Ca^{2+} channels following treatment with NO and a cGMP donor (Yoshimura et al., 2001). If BoNT/A (0) can directly increase intracellular concentrations of NO, the result may be direct and indirect inhibition of afferent mechanosensitivity.

The purinergic pathway was also investigated using the P2X antagonist TNP-ATP and assessed its effect on BoNT/A (0) induced afferent inhibition. When applied alone, TNP-ATP did not alter distension induced afferent firing, a finding also reported by Yu *et al* (2008) who found both TNP-ATP and the non-selective purinergic antagonist PPADS did not influence afferent firing (Yu & de

Groat, 2008). This may be a controversial finding due to the well described importance of ATP on sensory signalling in the bladder, due to previous literature finding altered afferent responses in mice without P2X receptors (Vlaskovska et al., 2001). Surprisingly, when TNP-ATP was co-applied with BoNT/A (0), the potent inhibition was also lost.

The similarity of response between antagonism of ATP and NO signalling was unexpected, however previous research in the cardiac field has shown purinergic receptors capable of modulating eNOS activity. Yamamoto *et al* (2006) found that genetic knockout of the P2X4 receptor in mice led to reduced mechanosensation in vascular endothelial cells in response to shear stress (Yamamoto et al., 2006). They reported that the loss of P2X4 dependent Ca^{2+} influx led to reduced NO release, which was rescued upon adenovirus gene transfer of P2X4 to cultured endothelial cells. Other studies have shown structural changes in the vasculature of P2X4 deficient mice similar to those found in eNOS deficient mice, such as increased wall thickness and loss of flow dependent changes in vascular diameter (Rudic et al., 1998; Yamamoto et al., 2006). Yang *et al* (2015) showed direct interactions between P2X4 and eNOS, finding colocalization as well as direct interaction through immunoprecipitation (Yang et al., 2015). They also found that activating P2X4 increased NO release in cardiac myocytes (Yang et al., 2015). P2X receptor dependent modulation of NO release does not appear to be restricted to the vasculature, this phenomenon has been observed in astrocytes, hippocampal neurons, and immune cells (Codocedo et al., 2013; Manohar et al., 2012; Murakami et al., 2003). These findings point to direct interactions between purinergic receptors and eNOS, showing that inhibition or loss of P2X receptors results in attenuated NO release (Yamamoto et al., 2006).

In light of these findings, a potential mechanism underlying the BoNT/A (0) function described in this chapter may be that it activates P2X receptors to stimulate NO release, as shown in figure 5.18 below. The result of this may be inhibition of bladder mechanosensitivity in different ways depending on the cell type of interest. BoNT/A (0) induced NO release may directly increase activation threshold of afferent nerves, reduce urothelial release of excitatory neurotransmitters and increase cGMP activity in interstitial cells. As previous studies investigating catalytically active BoNT/A have shown increased NO and decreased ATP release (Collins et al., 2013; Khera et al., 2004; Smith et al., 2005), there may be a common mechanism shared between them, and perhaps the mutations rendering BoNT/A (0) inactive made it more suitable for this function? While this is speculation at this point, further studies are now required to fully ratify this mechanism.

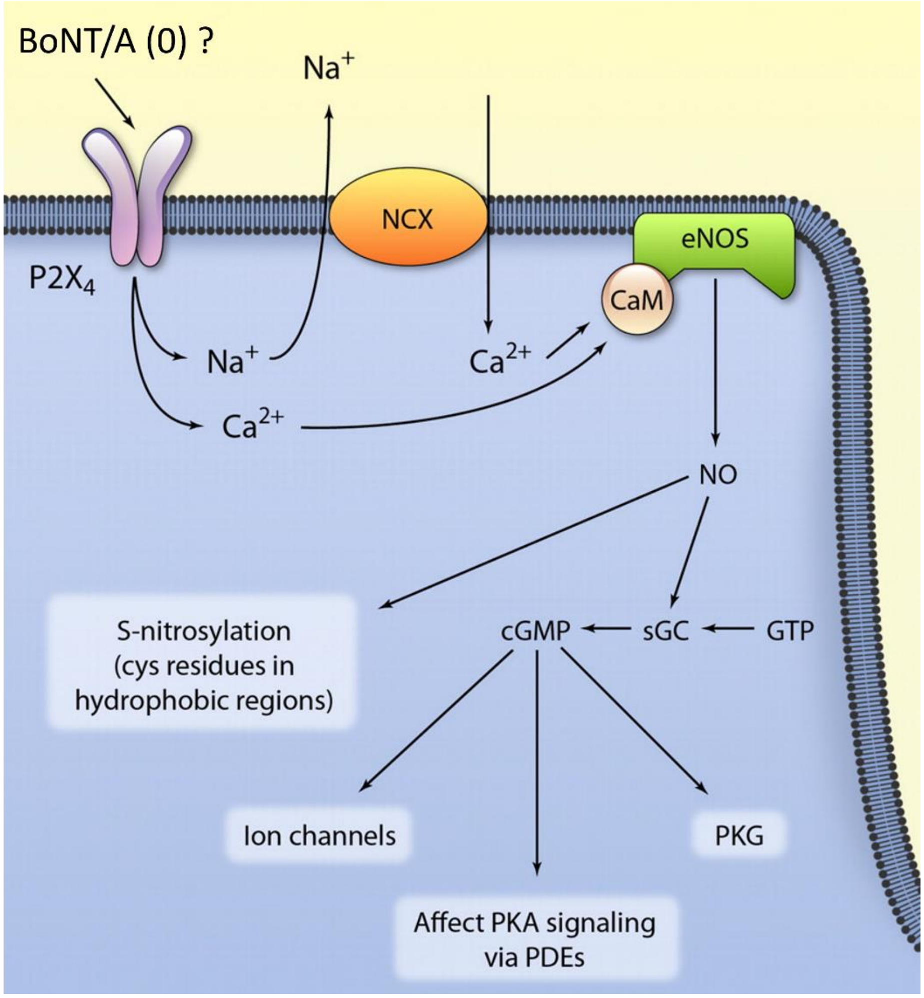


Figure 5.18: Proposed mechanism of BoNT/A (0) dependent modulation of bladder afferent mechanosensitivity. BoNT/A (0) may activate P2X4 on the cell membrane leading to an influx of Ca^{2+} within the cell, which binds calmodulin (CaM) to activate eNOS. The resultant NO produced may have

downstream effects through S-nitrosylation or activation of cGMP. NXC – a Na^+/Ca^{2+} exchanger, sGC – soluble guanylyl cyclase. Adapted from Yang & Liang (2012)

CONCLUSION

In this chapter, the effect of a novel recombinant BoNT/A construct without catalytic activity on bladder mechanosensitivity was characterised. This construct exerted significant effects on sensory signalling, a feature also exhibited by the light chain only (LC/A (0)) version, which supported the previous finding in chapter three that SV2 dependent binding was not necessary in facilitating the physiologic effect of afferent inhibition. Through pharmacological modulation of nitrergic and purinergic pathways, potential mechanisms underlying these effects were proposed. These data provide evidence that SNAP-25 cleavage activity may not be the driver behind the sensory effects of BoNT/A, which may provide a potential answer to the lack of cSNAP-25 immunoreactivity seen in chapter three. The implications of these findings are discussed in detail in the next chapter.

CHAPTER 6 – GENERAL DISCUSSION

This thesis details the results of an investigation of botulinum neurotoxins (BoNTs) and their effects on the transduction of sensory signals. This was achieved through the use of an *ex vivo* mouse bladder electrophysiology preparation and immunohistochemistry. The primary aim of this thesis was to explore the direct effects of BoNT serotype A on afferent neurotransmission that has previously been reported in the literature (Burstein et al., 2014; Collins et al., 2013; Zhang et al., 2016), as well as characterise other native and novel recombinant BoNTs.

6.1 Discovery of the sensory effects of catalytically inactive BoNT/A

BoNT/A has been shown to act directly on sensory neurotransmission, in the clinic it has been found to inhibit symptoms of disorders such as migraine (Barbanti et al., 2015; Burstein et al., 2014; Eross et al., 2005) and OAB (Binder et al., 2000; Chapple et al., 2013; Chuang et al., 2014; Craciun & Irwin, 2019; Dmochowski et al., 2010; Kuo et al., 2014). These effects may have been found by chance, as in the case of OAB the main rationale for the intravesical injection of BoNT/A was to inhibit hypercontractility of the detrusor smooth muscle (Schurch et al., 2000), with effects on the sensory symptom of urgency described later (Smith et al., 2002). In the field of migraine, researchers conducting clinical trials for the cosmetic use of Botox found patients reporting relief of migraine following forehead injections, which led them to design an open-label study investigating the potential prophylactic effect of BoNT/A on migraine. Binder *et al* (2000) found 51% of patients reporting improvements in symptoms which lasted 4.1 months on average (Binder et al., 2000). Relief from urgency following intravesical injection of BoNT/A has also been reported to be long lasting, with patients requiring repeat injection after 5-9 months (Mohee et al., 2013). There are other benefits supporting the use of BoNT/A as a treatment for sensory disorders other than its long duration of activity, most of which are the same as those reported for hypercontractility of muscle. It is a large molecule which does not readily disperse past where it is injected, which protects against non-specific effects.

The main reason behind the restricted use of BoNT/A in the clinic despite its efficacy in reducing aberrant sensory activation and nociception is its safety concerns. BoNT/A remains the most potent natural neurotoxin known to man, with a lethal dose of 1 ng/kg leading to restrictions of its handling and use. In this thesis, a catalytically inactive construct of BoNT/A (BoNT/A (0)) was characterised and its effect on afferent neurotransmission tested for the first time. The potent inhibition reported in chapter five was unexpected, as the mutation in the light chain prevented any cleavage of SNAP-25 which meant it was regarded as an inert molecule. Previous studies characterising other inactive versions of BoNT/A found no effects on acetylcholine release at the NMJ and no alterations in muscle contractility, showing a complete loss of toxicity (Baskaran et al., 2013; Ravichandran et al., 2015; Yang et al., 2008). These findings along with the data shown in chapter five support the conclusion that BoNT/A (0) lacks the safety concerns of active BoNT/A, has no effect on the NMJ nor on the contractile capacity of muscle, yet induces significantly more potent inhibition of sensory neurotransmission than active BoNT/A (see chapters three and five).

These findings support further development of BoNT/A (0) as a potential treatment for disorders of sensory signalling and nociception. The current landscape for the treatment of pain, especially chronic pain, is dire and leaves patients with little options other than non-opioid analgesics that may not be appropriate for long term use, or opioids which are associated with increased risk of addiction (Ballantyne & LaForge, 2007). The opioid crisis has spread worldwide and often people struggling with addiction were introduced to opioids through prescriptions following surgery or injury (Clarke et al., 2014). This illustrates the need for accelerated drug development of non-addictive analgesic drugs, which may play some part in stemming the growth of opioid-dependency in patients, which has a high risk of overdose and death (Stone et al., 2017).

Despite the large potential for benefit in the use of BoNT/A (0) for the treatment of pain, there are reasons for caution. At this stage, the only evidence pointing to sensory effects of BoNT/A (0) are based on a single experimental method. While at the close of this thesis the exact mechanism underlying the effect of BoNT/A (0) remains unclear, evidence in chapter five supports its modulation of purinergic and nitrergic pathways. Further investigations are necessary to confirm these findings and show the intracellular pathways underlying these effects. There is potential for BoNT/A (0) to modulate sensory responses to other stimuli such as chemical and inflammatory mediators. While there was no difference in responses to extravesical capsaicin, it remains unclear if responses to inflammation are altered. This may be investigated using *in vivo* models of inflammatory disorders such as the cyclophosphamide model of interstitial cystitis, which will show whether BoNT/A (0) attenuates inflammation induced hypersensitivity of afferent nerves as well as the duration of activity. This is an essential metric to investigate for the development of BoNT/A (0) as a viable treatment for sensory disorders, as it may not be financially or practically useful if any relief from pain subsides after a few hours.

The data collected in this project regarding BoNT/A (0) is novel and may be used as a springboard for further development, as there is a severe need for safe, effective, and potentially long-lasting treatment of pain without risk of addiction.

6.2 Effects of catalytically inactive BoNT/A on bladder mechanosensation

The consistent reports of relief from symptoms of urgency made by patients receiving BoNT/A treatment for OAB suggested a more complex mechanism than what was apparent previously, that the targeted silencing of cholinergic neurotransmission could not adequately explain. Investigating the direct effects of BoNTs on the detection and transduction of bladder stretch was made complicated by the fact that there are many open questions and gaps in our understanding regarding this topic. The favoured approach in exploring these mechanisms was to use a wide variety of BoNT constructs, mutants and serotypes and characterise their effects on afferent responses.

The maintenance of micturition is dependent on the healthy and consistent function of these sensory patterns, as integration of afferent signals provides input to the CNS that informs decisions concerning voiding. The primary afferent neurons are responsible for the transmission of the state of bladder fullness, graded distension leading to the activation of stretch sensitive fibres. The bladder is innervated with Aδ and C fibres, which have commonly been attributed to detect low threshold and high threshold stimuli respectively. This purported system connects bladder volume with perception, as the thresholds of additional classes of neurons are breached, allowing the sensation to progress to bladder fullness, discomfort and potentially pain.

There are gaps in our understanding regarding mechanisms underlying this process, which makes the study of the effects of BoNTs on bladder sensation less straightforward. Currently, there are two main hypotheses that attempt to explain the transduction of mechanical stretch to neural signals in the bladder wall, directly through activation of stretch sensitive channels, and indirectly through detection of neurotransmitters and sensory mediators released by the urothelium (Apodaca et al., 2007; Zagorodnyuk et al., 2006). BoNT/A has been implicated in the inhibition of both function and expression of stretch sensitive receptors (Apostolidis et al., 2005; Fan et al., 2017; Shimizu et al., 2012; Xiao et al., 2013; Zhang et al., 2016), as well as the release of mediators from the urothelium (Collins et al., 2013; Hanna-Mitchell et al., 2015; Khera et al., 2004; Smith et al., 2005). These functions have primarily been attributed to the targeting and cleavage of SNAP-25, presumably in the urothelial cells and the afferent nerve terminals, stopping the exocytotic mechanisms that mediate release of neurotransmitters and insertion of receptors into the plasma membrane. This may consequently interrupt nociceptive signalling and development of neurogenic inflammation.

To investigate the involvement of SNAP-25 cleavage in the inhibition of distension induced afferent firing following intravesical BoNT/A, BoNT/A (0), a mutated construct of BoNT/A lacking the ability to cleave SNAP-25 was tested. Prior to this experiment, the hypothesis developed was that the BoNT/A (0) protein would have a minor effect due to the loss of cleavage activity. Unexpectedly, intravesical treatment of BoNT/A (0) had an extremely potent inhibitory effect on the distension induced afferent response. These results may even indicate that ability to cleave SNAP-25 was a disadvantage to the active BoNT/A, as the only difference between the two proteins is a substitution of two amino acids (E224Q and H227Y). However, this is stated with caution as it was not possible to show SNAP-25 cleavage in the Dysport treated bladders (chapter three), so it cannot be said with certainty that active BoNT/A is able to cleave SNAP-25 in the present preparation. It is possible that the light chain mutation present in BoNT/A (0) led to a conformational change that increased its affinity for a non-SNAP-25 target, which explains the vast difference in physiological effect induced by two very similar proteins.

There are potential benefits to the use of BoNT/A (0) in the urology clinic. One of the major advantages of using catalytically inactive BoNT/A is the lack of paralytic activity, a previous in vivo mouse toxicology assay reported a lethal dose 1.2 million-fold higher than the minimum lethal dose of native BoNT/A, which suggests significantly improved safety margins (Ravichandran et al., 2015). In the treatment of OAB, injection of active BoNT/A at higher concentrations has an increased risk of side effects such as bladder retention. OAB patients who report the symptom of bladder retention require clean intermittent catheterisation (CIC), Karsenty et al (2014) reported between 12-19% of idiopathic OAB patients who received 300U intravesical BoNT/A injections started CIC (Karsenty et al., 2014). Due to its inability to cleave SNAP-25 and induce muscle paralysis, injection of BoNT/A (0) may provide the relief of urgency symptoms without the risk of bladder retention, which avoids the reduction in quality of life caused by the necessity of catheterisation.

The finding that a catalytically inactive construct of BoNT/A may have potent inhibition of sensory signalling could provide an option for the treatment of urgency and frequency that does not impact the ability of the detrusor to contract and initiate voiding. However, the extent to which BoNT/A

(0) inhibited sensory signalling could also be a disadvantage, as it could possibly reduce the ability of the afferents to detect bladder fullness to the extent that it leads to overdistension and damage to structures in the bladder wall. In this study, concentrations as low as 1 fM showed significant inhibition of distension induced firing, exemplifying the serious potency of BoNT/A (0) which suggests caution in its further testing and use.

6.3 Implications of BoNT/A entry without the double receptor mechanism

The double-receptor mechanism of BoNT/A entry into cholinergic neurons has been widely discussed and reproduced in the literature (Couesnon et al., 2007; Dong et al., 2006; Rummel et al., 2011). Reports of BoNT/A being unable to enter cells that do not express SV2 or the required gangliosides have led to the understanding that the specific targeting of cholinergic neurons depends on their expression of these target receptors. In chapter three, the primary aim was to investigate the entry mechanisms of BoNT/A, through the use of the BoNT/A complex (Dysport), as well as the individual subdomains (HC/A and LC/A).

The main finding in chapter three was that the only domain necessary to inhibit distension induced afferent firing was the LC/A domain, and that the other subdomains (HC_C and HC_N; receptor binding and translocation domains) were not necessary in the entry of BoNT/A. This data stands in contrast with the well-characterised double-receptor mechanism, where gangliosides and SV2 proteins are required for BoNT/A internalisation. The apparent lack of necessity of the translocation domain was also unexpected, as it was unclear how the LC/A domain may enter the cytosol even if it was endocytosed. This may be further investigated using fluorescent tags conjugated onto the LC/A protein, tracking its movement through cells. It is also unclear how the LC/A protein directly inhibited distension induced afferent firing, however, this question is not unique to the LC/A protein and is shared by all the BoNT constructs investigated in this study.

The fact that the LC/A domain is shared by all constructs that had any efficacy in reducing afferent firing provides confidence that this is not a non-specific activity that occurs with all exogenous proteins. Indeed, the preparations that received intravesical HC/A retained afferent responses throughout the experiment. While the main method utilised in this thesis provided a large amount of information regarding intravesical pressure and afferent firing, including simultaneous capture of multiple single units with individual response thresholds, it did not facilitate targeted study of cellular mechanisms that may be required to fully understand the specific actions of LC/A. Further cell-based studies may provide further insight to the function of all the BoNT/A constructs investigated in this study.

The exact mechanism by which BoNT/A may inhibit sensory activity is unclear. The cleavage of SNAP-25 has been used throughout the literature as a reliable end point to describe BoNT/A function, in a variety of cell types from neuronal to non-neuronal (Hanna-Mitchell et al., 2015; Huang et al., 2001; Ma et al., 2014; Meng et al., 2007). This has been coupled to the loss of vesicular release mechanisms which requires a functional SNARE complex. However, it is difficult to ascribe this mechanism to BoNT/A activity unrelated to vesicular release of mediators, for example the excitability of sensory neurons. Previous researchers have focused upstream or downstream of the afferent nerve responses, for example, the release of mediators such as ATP which activate the afferent nerve terminal, or the release of CGRP from the nerve terminal following excitation (Hanna-Mitchell et al., 2015; Ikeda et al., 2012; Joussain et al., 2019; Meng et al., 2007). As both CGRP and, in part, ATP release occurs through vesicles, it is easy to imagine how cleavage of SNAP-25 could cause impairment of sensory signalling, resulting in inhibition of sensory neuron activation or the initiation of neurogenic inflammation. However, the question of whether BoNT/A can act directly on sensory neurons to inhibit excitation remains.

As the specific targeting of cholinergic neurons has been lost due to the lack of receptor binding region, it may be argued that the LC/A domain alone may improve safety margins while retaining sensory activity. However, this may result in reduced efficacy due to the inability to target specific cells, as well as the risk of unwanted non-specific activity or side effects. In the literature, a variety of delivery methods have been developed to adapt or improve the entry of the light chain of BoNT/A, including liposomes (Kuo et al., 2014; Paiva & Dolly, 1990), hydrogel (Krhut et al., 2016), conjugation with a variety of ligands (Chaddock et al., 2000; Fonfria et al., 2016) and the productions of chimeras using targeting domains of different toxins including other BoNTs (Wang et al., 2008), tetanus (Wang et al., 2012), cholera (Corrie, 2021) and anthrax (Yang et al., 2022). These studies aimed to reduce the risk of non-specific activity by targeting the light chain to their cells of interest. In chapter three, a form of retargeted BoNT/A, the Targeted Secretion Inhibitor conjugated to the TrkA receptor (TrkA-TSI) was also investigated. This protein was intended to target sensory neurons specifically, however appeared to have no effect in the present preparation. This may be due to the TrkA-TSI not being able to contact the sensory nerves directly, due to an inability to breach the urothelial barrier. Until it is understood where in the bladder wall the LC/A and other BoNT/A constructs act to induce sensory inhibition, at the urothelial level by stopping the distension-induced release of afferent mediators, or at the afferent level by inhibiting mechanosensory pathways, it may be difficult to develop BoNTs further for this purpose.

6.4 Sensory effects of the BoNT family

An aim of this thesis was to explore the effects of the other members of the BoNT family on bladder sensation One of the BoNTs chosen was BoNT/B, due to a previous report of direct action on sensory neurotransmission (Marino et al., 2014) and moderate efficacy on treating symptoms of idiopathic OAB (Dykstra et al., 2003; Ghei et al., 2005; Hirst et al., 2007). Another aim was to further characterise the effects of BoNT/E on visceral sensation, as it has been well studied in primary culture but perhaps not in a whole organ setting. Interestingly, the present study showed that both BoNTs /B and /E were capable of directly inhibiting distension induced afferent firing, inducing a more potent inhibition of sensory nerve activity than that of BoNT/A. This may have occurred due to a variety of reasons, including higher expression of the receptor responsible for cellular uptake or SNARE protein target, which may be better understood following further studies. Although it was not possible to show immunoreactivity of the cleaved form of SNAP-25 in chapter three, it may still be possible that modulation of SNAREs is the primary mechanism of action of BoNTs. The making of this conclusion is complicated by the finding in chapter five that BoNT/A (0) imposed significant attenuation of mechanosensation, despite lacking any catalytic activity. These data may be reconciled through further studies.

The fact that BoNT/B inhibited distension induced firing in the mouse bladder does not necessarily mean it is able to break the monopoly held by BoNT/A in the clinic. The lack of affinity of BoNT/B to the human version of its receptor synaptotagmin II are a setback to its therapeutic use. There have been efforts to engineer BoNT/B through recombinant methods to show increased affinity to human synaptotagmin II, Tao *et al* (2017) showed that substitution of a glutamic acid residue in the synaptotagmin II binding region improved affinity for human cells and subsequently increased cleavage of VAMP-2 (Tao et al., 2017). This mutant BoNT/B was further tested in preclinical studies in humanised mouse models and showed significantly higher efficacy compared to wild type BoNT/B (Elliott et al., 2019). These studies provide insight on how slight modifications to BoNTs that may appear unsuitable for clinical use can show improved efficacy and support further development and engineering of BoNT proteins.

6.5 Limitations

While the *ex vivo* bladder electrophysiology assay provided vast amounts of information regarding afferent responses to mechanical and chemical stimuli, further studies using a wider variety of techniques may have allowed us to develop a more detailed picture of the function of the BoNTs under investigation. For example, one of the major questions remaining at the end of this project is the cell type of interest to BoNTs in the inhibition of sensory signalling; the urothelium, the afferent nerve terminals, the interstitial cells, or another, less studied cell type that may be involved in mechanotransduction. A potential way to study the direct effects of BoNTs on afferent nerves is use protamine sulphate, a chemical agent widely used to remove the urothelial layer. Unfortunately, this experiment was unsuitable as it may have led to damage to the afferent terminals that innervate the urothelium and thus, made interpretation of results difficult.

The reliance on the *ex vivo* bladder preparation for the characterisation of BoNTs in this thesis may be construed as a disadvantage, as any data collected are based on short term recordings. This may have an impact on the translatability of the findings of this thesis, as it is not possible to comment on the long-term effects of the BoNT constructs investigated, and their suitability for use in treatment of bladder disorders. This limitation is particularly acute in the discussions on catalytically inactive BoNT/A (0) and LC/A (0), as they were characterised for the first time in this thesis. Further long-term, *in vivo* studies could shed light on whether the effects seen in chapter five persist longer than measured in the experimental conditions used here. These studies could include basic cystometry experiments staggered over days, to assess the suitability of BoNT/A (0) as a viable alternative to catalytically active BoNT/A. It is also important to conduct experiments in species other than mouse, to ensure the observed effects are not species specific.

The generation of concentration-response curves by testing multiple concentrations of each BoNT would have produced a more robust analysis and informed further studies. This was not possible to achieve in this study due to constraints in time and resources. Despite that, multiple concentrations of LHn/A (chapter three) and BoNT/A (0) (chapter five) were tested, which created a clearer picture on the functionality of these BoNTs. Further studies can be conducted to produce concentration response curves, perhaps using a higher thoroughput method such as calcium imaging of DRG neurons.

6.6 Future directions

In this thesis, a preliminary study on the effects of BoNTs on bladder sensory signalling was conducted, investigating many of these neurotoxins for the first time in the bladder. Although the experiments outlined in this study provide important information on BoNT modulation of distension induced afferent firing, there are many outstanding questions at the close of this project.

In the context of drug development, the most attractive candidate for continued experimentation and characterisation is the BoNT/A (0) protein due to its improved safety margins and apparent silencing of afferent signalling. In fact, further development may create two separate forms of BoNT/A with separate uses, one which cleaves SNAP-25 and used to paralyse muscle, and one which cannot cleave SNAP-25 and used to inhibit nociceptive signalling. The finding that the LC/A (0) molecule is also active at inhibiting sensory neurotransmission without affecting motor function may also prove it useful for further development, as it may be conjugated with targeting ligands that bind receptors expressed on sensory neurons. This would avoid any non-specific or unwanted effects and directs its potent activity to where it is needed. The other BoNTs tested in this thesis including the BoNT/B and BoNT/E proteins may also have potential in treating disorders of both motor and sensory origins.

In terms of the specific effects on the bladder, it remains unclear where in the bladder wall the BoNTs tested in this project act to induce sensory inhibition, and indeed it is possible that they have different mechanisms of actions or targets. This is clear in the comparison between catalytically active and inactive BoNT/A. This investigation may be hampered by the fact that the exact mechanism of mechanotransduction in the bladder is not clear at present, and if further studies are able to shed light on this process, it may inspire research that can unveil the exact mechanism of BoNT induced inhibition of afferent firing.

The *ex vivo* electrophysiology preparation can be used as a model to answer questions on visceral sensation more globally. The results contained in this thesis can inform studies on the effect of BoNTs on visceral sensation in other organ systems, especially in the context of chronic pain and hypersensitivity. An exciting prospect of BoNT/A action on afferent nerves is its potential effects on cross-organ sensitization. Early research on patients with irritable bowel syndrome (IBS) found that they also exhibit lower urinary tract symptoms including detrusor instability (Whorwell et al., 1986), while 31.2% of patients receiving treatment for urinary symptoms also had IBS (Alagiri et al., 1997; Francis et al., 1997). Cross-sensitization between the bladder, colon and reproductive organs has been reported clinically, interstitial cystitis and IBS has been shown to co-occur in some women with endometriosis (Berkley et al., 2005). These organs have converging sensory pathways which could provide some explanation for the comorbidities, afferent signalling from the pelvic organs (bladder, uterus, colon and rectum) is propagated through the hypogastric, pelvic, pudendal and splanchnic nerves (Berkley et al., 2005). The pathogenesis of cross-organ sensitization could be due to central processing, or peripheral processing due to dichotomizing fibres, which have nerve terminals in multiple tissues. Winnard *et al* (2006) showed that inducing inflammation in the colon or uterus through exposure to mustard oil led to plasma extravasation in the bladder, and cutting the hypogastric nerve alleviated this (Winnard et al., 2006). Bladder distension in rats pre-treated with dextran sulphate sodium induced (DSSI) colitis led to increased excitability of lumbosacral spinal neurons (Qin et al., 2005). Ustinova *et al* (2006) showed that colonic inflammation led to mechanical hypersensitivity of the bladder at high (>30 mmHg) pressures, and increased responses to chemical stimulation by capsaicin, bradykinin and substance P (Ustinova et al., 2006). These studies suggest that pain and inflammation can affect areas other than the insult.

This idea may be further investigated through the use on *in vivo* disease models such as the DSSI colitis model or cyclophosphamide induced cystitis, where BoNT/A or BoNT/A (0) is administered to an organ other than the one that received the insult. This could not only provide additional information regarding the neural control of sensory information in disease states, but also answer the question of whether BoNTs may directly modulate afferent sensitization and what effect this could potentially have on upstream processes. It is also important to better understand the long-term effects of these neurotoxins, especially BoNT/A (0), which could be tackled in these proposed *in vivo* studies.

6.7 Conclusion

In this thesis, a systematic analysis of the effects of BoNT on bladder sensory signalling was conducted, through the investigation of both native and recombinant forms. A wide array of BoNTs and their sub-fragments were studied using an experimental model that is physiologically relevant, in hopes to provide a springboard for future studies. The treatment options for patients with chronic sensory disorders such as pain and overactive bladder remains lacking, which constitutes a major problem as global populations age. As intravesical injection of BoNT/A has become a well-tolerated third line treatment with some side effects, it may be possible that further development of BoNTs, including use of other serotypes or testing of recombinant chimeras, may improve the quality of life for patients with OAB and other sensory disorders.

SUPPLEMENTARY DATA

To ensure the potent inhibitory effect of BoNT/A (0) was not dependent on batch effects, a different batch of BoNT/A (0) was tested in the *ex vivo* bladder-nerve preparation. The second batch of BoNT/A induced significant inhibition in distension induced afferent firing, as 53.23% (+/-15.84%; figure 6.1A) of afferent firing was remaining 90 minutes after exposure (p<0.0001; *n* = 3). The AUC analysis also revealed significant inhibition (p = 0.014; *n* = 3; figure 6.1B), which suggests the afferent inhibition induced by BoNT/A (0) is due to a feature of the molecule itself and not vulnerable to batch effects.

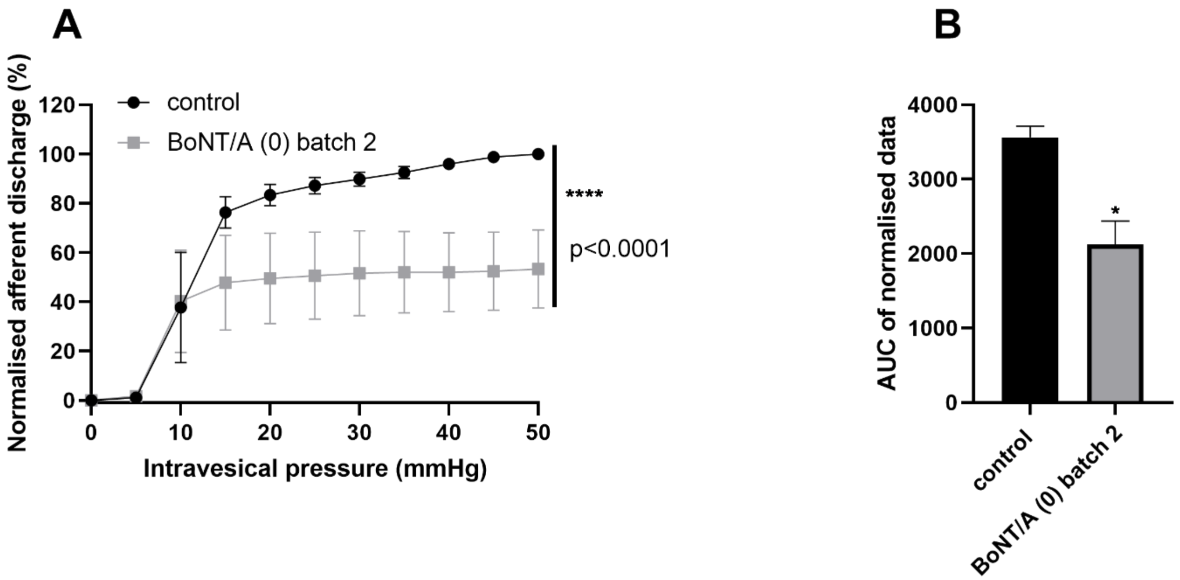


Figure 6.1: A second batch of BoNT/A (0) significantly inhibited afferent mechanosensitivity. **A)** Distension induced afferent firing was significantly inhibited by BoNT/A (0) batch 2 (p<0.0001; *n* = 3; two-way ANOVA). **B)** The AUC analysis also showed significant inhibition in afferent mechanosensitivity induced by treatment with BoNT/A (0) (p = 0.014; *n* = 3; T-test).

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